

FORM PTO-1390 (REV 12-29-99)		U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		ATTORNEY'S DOCKET NUMBER 45300-59676
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		U.S. APPLICATION NO. (If known, see 37 CFR 1.5) 09/508967		PCT/PTO MAR 20 2000 PATENT & TRADEMARK OFFICE
INTERNATIONAL APPLICATION NO. PCT/SE98/01675	INTERNATIONAL FILING DATE 18 SEPTEMBER 1998	PRIORITY DATE CLAIMED 19 SEPTEMBER 1997		
TITLE OF INVENTION MALARIA POLYPEPTIDES				
APPLICANT(S) FOR DO/EO/US Mats WAHLGREN, Antonio BARRAGAN, Jöhan CARLSON Chen QIJUN and Victor FERNANDEZ				
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:				
<p>1. <input checked="" type="checkbox"/> This is a FIRST submission of items concerning a filing under 35 U.S.C. 371.</p> <p>2. <input type="checkbox"/> This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371.</p> <p>3. <input checked="" type="checkbox"/> This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).</p> <p>4. <input checked="" type="checkbox"/> A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.</p> <p>5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371(c)(2)) <ul style="list-style-type: none"> a. <input checked="" type="checkbox"/> is transmitted herewith (required only if not transmitted by the International Bureau). b. <input type="checkbox"/> has been transmitted by the International Bureau. c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US). </p> <p>6. <input type="checkbox"/> A translation of the International Application into English (35 U.S.C. 371(c)(2)).</p> <p>7. <input type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)) <ul style="list-style-type: none"> a. <input type="checkbox"/> are transmitted herewith (required only if not transmitted by the International Bureau). b. <input type="checkbox"/> have been transmitted by the International Bureau. c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired. d. <input type="checkbox"/> have not been made and will not be made. </p> <p>8. <input type="checkbox"/> A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).</p> <p>9. <input type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).</p> <p>10. <input type="checkbox"/> A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).</p>				
Items 11. to 16. below concern document(s) or information included:				
<p>11. <input type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98.</p> <p>12. <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.</p> <p>13. <input checked="" type="checkbox"/> A FIRST preliminary amendment. <input type="checkbox"/> A SECOND or SUBSEQUENT preliminary amendment.</p> <p>14. <input type="checkbox"/> A substitute specification.</p> <p>15. <input type="checkbox"/> A change of power of attorney and/or address letter.</p> <p>16. <input checked="" type="checkbox"/> Other items or information: Sequence Listing (10 pages) International Preliminary Examination Report International Search Report Patent Data Entry Sheet</p>				

U.S. APPLICATION NO. (if known, see 37 CFR 1.5)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER	
09/508967		PCT/SE98/01675 45300-59676	
<p>17. <input checked="" type="checkbox"/> The following fees are submitted:</p> <p>BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :</p> <p>Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO \$970.00</p> <p>International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO \$840.00</p> <p>International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO \$690.00</p> <p>International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) \$670.00</p> <p>International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) \$96.00</p>		CALCULATIONS PTO USE ONLY	
ENTER APPROPRIATE BASIC FEE AMOUNT =		\$ 970	
<p>Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input checked="" type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(e)).</p>		\$ 130	
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE
Total claims	32 - 20 =	12	X \$18.00
Independent claims	3 - 3 =	0	X \$78.00
MULTIPLE DEPENDENT CLAIM(S) (if applicable)		+ \$260.00	
TOTAL OF ABOVE CALCULATIONS =		\$ 1,316	
Reduction of 1/2 for filing by small entity, if applicable. A Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28).		\$	
SUBTOTAL =		\$ 1,316	
Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f)).		\$	
TOTAL NATIONAL FEE =		\$ 1,316	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property		+	
TOTAL FEES ENCLOSED =		\$ 1,316	
		Amount to be refunded:	\$
		charged:	\$
<p>a. <input checked="" type="checkbox"/> A check in the amount of <u>\$ 1,316.</u> to cover the above fees is enclosed.</p> <p>b. <input type="checkbox"/> Please charge my Deposit Account No. _____ in the amount of \$_____ to cover the above fees. A duplicate copy of this sheet is enclosed.</p> <p>c. <input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge any additional fees which may be required by 37 CFR §1.16 and 1.17, or credit any overpayment to Deposit Account No. 25-0120. A duplicate copy of this sheet is enclosed.</p>			
<p>NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.</p>			
SEND ALL CORRESPONDENCE TO: YOUNG & THOMPSON 745 South 23rd Street Second Floor Arlington, VA 22202 CUSTOMER NO. 000466 Tel. (703)521-2297 Fax (703)685-0573		March 20, 2000  SIGNATURE: Benoit Castel NAME 35,041 REGISTRATION NUMBER	

09/508967
422 Rec'd PCT/PTO 20 MAR 2000

PATENTS

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of

Mats WAHLGREN et al.

Serial No. (unknown)

Filed herewith

MALARIA POLYPEPTIDES

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents

Washington, D.C. 20231

Sir:

Prior to the first Official Action and calculation of the filing fee, please amend the above-identified application as follows:

IN THE CLAIMS:

Claim 4, line 1, change "any one of claims 1-3," to
--claim 1,--.

Claim 5, line 1, change "any one of claims 1-4," to
--claim 1,--.

Claim 6, line 1, change "any one of claims 1-5," to
--claim 1,--.

Claim 7, line 1, change "any one of claims 1-6," to
--claim 1,--.

Claim 8, line 1, change "any one of claims 1-7," to
--claim 1,--.

Claim 9, line 1, change "any one of claims 1-7," to
--claim 1,--.

Claim 10, lines 1 and 2, change "any one of claims 1-7," to --claim 1,--.

Claim 15, line 1, cancel "or 14".

Claim 16, line 1, change "any one of claims 13-15," to --claim 13,--.

Claim 17, line 1, change "any one of claims 13-16," to --claim 13,--.

Claim 18, line 1, change "any one of claims 13-17," to --claim 13,--.

Claim 19, line 1, change "any one of claims 13-17," to --claim 13,--.

Claim 21, lines 1 and 2, change "any one of claims 13-17," to --claim 13,--.

Claim 22, line 1, change "any one of claims 13-17," to --claim 13,--.

Claim 23, line 1, change "any one of claims 13-17," to --claim 13,--.

Claim 24, line 1, change "any one of claims 13-17," to --claim 13,--.

Claim 27, line 1, change "any one of claims 16-18," to --claim 16,--.

Claim 28, line 2, change "any one of claims 13-17," to --claim 13,--.

Claim 32, line 1, cancel "or 31".

Mats WAHLGREN et al.

R E M A R K S

The above changes in the claims merely place this national phase application in the same condition as it was during Chapter II of the international phase, with the multiple dependencies being removed.

Respectfully submitted,

YOUNG & THOMPSON

By


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March 20, 2000

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MALARIA POLYPEPTIDESTechnical field

The present invention relates to carbohydrates capable of acting as receptors for malaria antigens present on the surfaces of malaria infected erythrocytes. In addition, the invention also relates to novel malaria polypeptides capable of acting as ligands in relation to the receptors according to the invention. The invention also encompasses the use thereof as medicaments and vaccines, pharmaceutical compositions containing the same as well as antibodies directed against said new ligands.

Background and prior art

On a worldwide basis, malaria is one of the most common infectious diseases. Even though it has been largely eliminated from North America and Europe, it remains the most serious infectious disease in tropical and subtropical regions of the world.

According to the World Health Organisation, WHO, there are about 100 million new cases each year, and about 300 million people in the developing countries exhibits chronic malaria infections. Malaria is caused by four species of *Plasmodium*, of which *Plasmodium vivax* and *Plasmodium falciparum* are most frequently involved in human infections. The vector responsible for transmitting malaria to humans is the Anopheles mosquito and only malaria infections caused by *Plasmodium falciparum* may be fatal to humans.

After inoculation in the body, the sporozoites of *Plasmodium* begin to reproduce within liver cells. Multiplication of *Plasmodium* sporozoites occurs by schizogony, in which a single sporozoite can produce as many as 40,000 merozoites. The invasion of erythrocytes by hepatic merozoites begins the erythrocytic phase of malaria, causing fever and other severe manifestations.

Erythrocytes infected with the malaria parasite *P. falciparum* disappear from the peripheral circulation as they mature from the ring stage to trophozoites. This

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Ma, M. Feldman, T.F. Taraschi, and R.J. Howard. 1995. Cloning the *P. falciparum* gene encoding PfEMP1, a malaria variant antigen and adherence receptor on the surface of parasitized human erythrocytes. *Cell* 82, 77-87). The feature of antigenic variation and switching of the surface of the pRBC has been attributed to the *var*-genes. Even though up to 150 such genes are harboured in the genome, only one PfEMP1 is thought to be expressed at any one time. PfEMP1 has features of an adhesive molecule and has been associated with the cytoadherent properties of the infected red cell (Smith, J. D., C.E. Chitnis, A.G. Craig, D.J. Roberts, D.E. Hudson-Taylor, D.S. Peterson, R. Pinches, C.I. Newbold, and L.H. Miller. 1995. Switches in expression of *Plasmodium falciparum* *var* genes correlate with changes in antigenic and cytoadherent phenotypes of infected erythrocytes. *Cell* 82, 101-110, Baruch, D. I., J.A. Gormley, C. Ma, R.J. Howard, and B.L. Pasloske. 1996. *Plasmodium falciparum* erythrocyte membrane protein 1 is a parasitised erythrocyte receptor for adherence to CD36, thrombospondin, and intercellular adhesion molecule 1. *Proc. Natl. Acad. Sci. U.S.A.* 93, 3497-3502). For example, the expression of PfEMP1-encoding *var* genes has been shown to correlate with the capacity of the pRBC for binding to host receptors, including CD36 and ICAM-1 (Baruch, D. I., J.A. Gormley, C. Ma, R.J. Howard, and B.L. Pasloske. 1996. *Plasmodium falciparum* erythrocyte membrane protein 1 is a parasitised erythrocyte receptor for adherence to CD36, thrombospondin, and intercellular adhesion molecule 1. *Proc. Natl. Acad. Sci. U.S.A.* 93, 3497-3502, Gardner, J.P., R.A. Pinches, D.J. Roberts, and C.I. Newbold, (1996). Variant antigens and endothelial receptor adhesion in *Plasmodium falciparum*. *Proc. Natl. Acad. Sci. U.S.A.* 93, 3503-3508). A role for PfEMP1 in rosetting was recently suggested by Rowe *et al* (Rowe, J.A., J.M. Moulds, C.I. Newbold, and L.H. Miller. 1997. *P. falciparum* rosetting mediated by a parasite-variant erythrocyte membrane protein and complement-receptor 1. *Nature* 388:292-295). Complement receptor 1 was found to be the host-receptor. However, it is believed that it cannot be the only one used by rosetting parasites.

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In WO 96/33736, one PfEMP1 variant is disclosed by sequence. However, this is only one of several possibly existing variants, all of which are different as to functional sequences as well as adhesive properties.

5 Accordingly, due to the complex nature and/or mechanism of malarial antigenic variation and the virulence thereof, there is still a great need for methods and compositions which may be useful in the treatment, diagnosis and prevention of malaria infections.

10 Object of the invention

The present invention fulfills the need described above by providing a carbohydrate, which is useful as a receptor for malaria erythrocyte membrane protein. The invention also provides such a new malaria protein acting as a ligand in relation to the receptors according to the invention.

15 Brief description of the drawings

Figure 1 discloses the identification of rosetting PfEMP1.

Figure 2A-C disclose, respectively, maps of cDNA structure, sequencing clones, deduced amino acid sequence and the location of GAG binding motifs in the rosetting PfEMP1 of FCR3S1.2.

20 Figure 3 discloses the binding of rosetting FCR3S1.2- PfEMP1 to heparine as seen by gel analysis of 10% SDS-PAGE stained with Coomassie.

Figure 4 discloses the disruption of preformed natural rosettes with either one of the fusion proteins DBL-I-GST or ATS-GST.

25 Figure 5 discloses the effect of glycosaminoglycans on *Plasmodium falciparum* rosetting.

Definitions

The term "glycosaminoglycan-like" as used in the present disclosure refers to any molecule or substance, that exhibits the same or similar advantageous properties as the

glycosaminoglycans comprising repeated units of sulfated or acetylated disaccharides and further discussed below.

As used herein, the term "essentially specific binding" in the context of the carbohydrates according to the invention is interpreted as such a specific binding as is needed to influence the rosetting of malaria erythrocyte membrane protein without having any other notable impact on the environment.

The terms "polypeptide", "peptide" and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to amino acid polymers, in which one or more amino acid residue is an artificial chemical analogue of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers.

The terms "isolated", "purified" or "biologically pure" refer to material which is substantially or essentially free from components which normally accompany it as found in its native state.

The term "biologically active fragment" as used herein refers to portions of the proteins or polypeptides possessing a particular biological activity, e.g. one or more activities found in the full length novel PfEMP1 variant according to the invention.

The term "fusion protein" as used herein generally refers to a composite protein made up of two or more separate, heterologous proteins.

The term "substantial identity" or "substantial similarity" in the context of a polypeptide indicates that a polypeptides comprises a sequence with at least 70% sequence identity to a reference sequence, or preferably 80%, or more preferably 85% sequence identity to the reference sequence, or most preferably 90% identity over a comparison window of about 10-20 amino acid residues. An indication that two polypeptide sequences are substantially identical is that one peptide is immunologically reactive with antibodies raised against the

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second peptide. Thus, a polypeptide is substantially identical to a second polypeptide, for example, where the two peptides differ only by a conservative substitution.

A "conservative substitution", when describing a protein, refers to a change in the amino acid composition of the protein that does not substantially alter the protein's activity.

Thus, "conservatively modified variations" of a particular amino acid sequence refers to amino acid substitutions of those amino acids that are not critical for protein activity or substitution of amino acids with other amino acids having similar properties (e.g., acidic, basic, positively or negatively charged, polar or non-polar, etc.) such that the substitutions of even critical amino acids do not substantially alter activity. Conservative substitution tables providing functionally similar amino acids are well known in the art. (See, e.g., Creighton (1984) *Protein*, W.H. Freeman and Company.) In addition, individual substitutions, deletions or additions which alter, add or delete a single amino acid or a small percentage of amino acids in an encoded sequence are also "conservatively modified variations".

An indication that two nucleic acid sequences are substantially identical is that the polypeptide which the first nucleic acid encodes is immunologically cross reactive with the polypeptide encoded by the second nucleic acid.

Another indication that two nucleic acid sequences are substantially identical is that the two molecules hybridize to each other under stringent conditions, as discussed in more detail below.

The term "nucleic acid" refers to a deoxyribonucleotide or ribonucleotide polymer in either single- or double-stranded form, and unless otherwise limited, encompasses known analogues of natural nucleotides that can function in a similar manner as naturally occurring nucleotides.

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The phrase "hybridizing specifically to", refers to the binding, duplexing, or hybridizing of a molecule only to a particular nucleotide sequence under stringent conditions when that sequence is present in a complex mixture (e.g., total cellular) DNA or RNA. The term "stringent conditions" refers to conditions under which a probe will hybridize to its target subsequence, but to no other sequences. Stringent conditions are sequence-dependent and will be different in different circumstances. Longer sequences hybridize specifically at higher temperatures. Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point T_m for the specific sequence at a defined 5 ionic strength and pH. The T_m is the temperature (under defined ionic strength, pH, and nucleic acid concentration) at which 50% of the probes complementary to the target sequence hybridize to the target sequence at equilibrium. (As the target sequences are generally present in excess, at T_m , 50% of the probes are occupied at equilibrium). 10 Typically, stringent conditions will be those in which the salt concentration is less than about 1.0 M Na ion, typically about 0.01 to 1.0 M Na ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C for short probes (e.g., 10 to 50 nucleotides) and at least about 60°C for long probes (e.g., greater than 50 nucleotides). 15 Stringent conditions may also be achieved with the addition of destabilizing agents, such as formamide.

20 As used herein a "nucleic acid probe" is defined as a nucleic acid capable of binding to a target nucleic acid of complementary sequence through one or more types of chemical bonds, usually through complementary base pairing, usually through hydrogen bond formation. As used herein, a probe may include natural (i.e. A, G, C, or T) or modified bases (7-deazaguanosine, inosine, etc.) In addition, the bases in a probe may be joined by 25 a linkage other than a phosphodiester bond, so long as it does not interfere with hybridization. Thus, for example, probes may be peptide nucleic acids in which the constituent bases are joined by peptide bonds rather than phosphodiester linkages. It will be understood by one of skill in the art that probes may bind target sequences lacking complete complementarity with the probe sequence depending upon the stringency of the 30 hybridization conditions. The probes are preferably directly labelled as with isotopes,

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chromophores, lumiphore, chromogens, or indirectly labelled such as with biotin to which a streptavidin complex may later bind. By assaying for the presence or absence of the probe, one can detect the presence or absence of the select sequence or subsequence.

5 A "label" is a composition detectable by spectroscopic, photochemical, biochemical, immunochemical, or chemical means. For example, useful labels include ^{32}P , fluorescent dyes, electron-dense reagents, enzymes (e.g., as commonly used in a ELISA), biotin, dioxigenin, or haptens and proteins for which antisera or monoclonal antibodies are available (e.g., the peptide of the invention can be made detectable, e.g., by incorporating a radio-label into the peptide, and used to detect antibodies specifically reactive with the peptide).

10 15 A "labelled nucleic acid probe" is a nucleic acid probe that is bound, either covalently, through a linker, or through ionic, van der Waals or hydrogen bonds to a label such that the presence of the probe may be detected by detecting the presence of the label bound to the probe.

20 The term "target nucleic acid" refers to a nucleic acid (often derived from a biological sample), to which a nucleic acid probe is designed to specifically hybridize. It is either the presence or absence of the target nucleic acid that is to be detected, or the amount of the target nucleic acid that is to be quantified. The target nucleic acid has a sequence that is complementary to the nucleic acid sequence of the corresponding probe directed to the target. The term target nucleic acid may refer to the specific subsequence of a larger nucleic acid to which the probe is directed or to the overall sequence (e.g., gene or 25 mRNA) whose expression level it is desired to detect. The difference in usage will be apparent from context.

"Bind(s) substantially" refers to complementary hybridization between a probe nucleic acid and a target nucleic acid and embraces minor mismatches that can be accommodated

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by reducing the stringency of the hybridization media to achieve the desired detection of the target polynucleotide sequence.

5 "Subsequence" refers to a sequence of nucleic acids or amino acids that comprise a part of a longer sequence of nucleic acids or amino acids (e.g., polypeptide), respectively.

In the present disclosure, the term "antibody" refers to a polypeptide substantially encoded by an immunoglobulin gene or immunoglobulin genes, or fragments thereof which specifically bind and recognize an analyte (antigen). The recognized immunoglobulin genes include the kappa, lambda, alpha, gamma, delta, epsilon and mu constant region genes, as well as the myriad immunoglobulin variable region genes. Light chains are classified as either kappa or lambda. Heavy chains are classified as gamma, mu alpha, delta, or epsilon, which in turn define the immunoglobulin classes, IgG, IgM, IgA, IgD and IgE, respectively.

10 15 A "chimeric antibody" is an antibody molecule in which (a) the constant region, or a portion thereof, is altered, replaced or exchanged so that the antigen binding site (variable region) is linked to a constant region of a different or altered class, effector function and/or species, or an entirely different molecule which confers new properties to the chimeric antibody, e.g., an enzyme, toxin, hormone, growth factor, drug, etc.; or (b) the variable region, or a portion thereof, is altered, replaced or exchanged with a variable region having a different or altered antigen specificity.

20 25 The term "immunoassay" is an assay that utilizes an antibody to specifically bind an analyte. The immunoassay is characterized by the use of specific binding properties of a particular antibody to isolate, target, and/or quantify the analyte.

30 The phrases "specifically binds to a protein" or "specifically immunoreactive with", when referring to an antibody refers to a binding reaction which is determinative of the presence of the protein in the presence of a heterogeneous population of proteins and

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other biologics. Thus, under designated immunoassay conditions, the specified antibodies bind preferentially to a particular protein and do not bind in a significant amount to other proteins present in the sample. Specific binding to a protein under such conditions requires an antibody that is selected for its specificity for a particular protein. A variety of immunoassay formats may be used to select antibodies specifically immunoreactive with a particular protein. For example, solid-phase ELISA immunoassays are routinely used to select monoclonal antibodies specifically immunoreactive with a protein. See Harlow and Lane (1988) *Antibodies, A Laboratory Manual*, Cold Spring Harbour Publications, New York, for a description of immunoassay formats and conditions that can be used to determine specific immunoreactivity.

The term "recombinant" when used with reference to a cell, or nucleic acid, or vector, indicates that the cell, or nucleic acid, or vector, has been modified by the introduction of a heterologous nucleic acid or the alteration of a native nucleic acid, or that the cell is derived from a cell so modified. Thus, for example, recombinant cells express genes that are not found within the native (non-recombinant) form of the cell or express native genes that are otherwise abnormally expressed, under expressed or not expressed at all. The term "identical" in the context of two nucleic acids or polypeptide sequences refers to the residues in the two sequences which are the same when aligned for maximum correspondence. Optimal alignment of sequences for comparison can be conducted, e.g., by the local homology algorithm of Smith and Waterman (1981) *Adv. Appl. Math.* 2: 482, by the homology alignment algorithm of Needleman and Wunsch (1970) *J. Mol. Biol.* 48:443, by the search for similarity method of Pearson and Lipman (1988) *Proc. Natl. Acad. Sci. USA* 85: 2444, by computerized implementations of these algorithms (GAP, GESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI) or by inspection. An additional algorithm that is suitable for determining sequence similarity is the BLAST algorithm, which is described in Altschul *et al.* (1990) *J. Mol. Biol.* 215: 403-410. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>).

Detailed description of the present invention

The present invention is disclosed in more detail below. All references throughout the present application are hereby included herein by references.

5

Thus, in a first aspect, the present invention relates to a carbohydrate, which exhibits at least one negatively charged glycosaminoglycan-like moiety and thereby is capable of essentially specific binding to a malaria erythrocyte membrane protein or an analogue thereof. Accordingly, the carbohydrate according to the invention may be used as a receptor to bind ligands, which are expressed on the surface of cells infected by malaria, such as erythrocytes infected by *Plasmodium falciparum*. In a preferred embodiment, said glycosaminoglycan moiety is sulfated and more specifically, it may be a heparan sulfate moiety. Most preferably, the carbohydrate according to the invention is fucoidan, or a functional fragment thereof. Further, the invention also relates to any functionally equivalent analogues of the above described carbohydrates.

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Indeed, it has been shown in the prior art that *P. falciparum*-rosettes may be disrupted by low concentrations of heparin, an effect that is immediate and reversible (Carlson, J., H.P. Ekre, H. Helmby, J. Gysin, B.M. Greenwood, and M. Wahlgren. 1992.

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Disruption of *Plasmodium falciparum* erythrocyte rosettes by standard heparin and heparin devoid of anticoagulant activity. *Am. J. Trop. Med. Hyg.* 46, 595-602, Rowe, A., A.R. Berendt, K. Marsh, and C.I. Newbold. 1994. *Plasmodium falciparum*: a family of sulfated glycoconjugates disrupts erythrocyte rosettes. *Exp. Parasitol.* 79, 506-16). However, prior to the present invention, it has not been possible to explain the mechanism or basis for this phenomena and, accordingly, it has not been possible to create any such advantageous and useful receptors as the present receptor carbohydrate until now. Thus, for the first time, the present invention discloses receptors capable of essentially specific binding of such malaria proteins.

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The glycosaminoglycans, or GAGs, being the receptor carbohydrate according to the invention, are composed of repeated units of sulfated or acetylated di-saccharides and are present in the human body bound to a protein-core in the form of proteoglycans (PG) (Yanagisha, M. and V. Hascall. 1992. Cell surface heparan sulfate proteoglycans.

5 *J. Biol. Chem.* 267, 9451-9454). Heparan sulfate and chondroitin sulfate are GAGs exposed at all human cell-surfaces, but they are diverse and vary from cell-to-cell and maybe within the same cell due to secondary modifications of the extensive carbohydrate chains (deacetylations, O-sulfations etc.). Heparin is only found in mast-cells and is characterized by a higher degree of sulfation and epimerization than heparan sulfate. However, heparan sulfate and heparin are similar due to heparin-like stretches also found in heparan sulfate (Faham,S., E.R. Hileman, R.J. Fromm, J.R. Lindardt, and C.D. Rees. 1996. Heparin structure and interactions with basic fibroblast growth factor. *Science*, 271, 1116-1120).

15 HS and heparin, both members of the heterogeneous GAG family, are composed of alternating glucosamine and uronic acid residues. The glucosamine can be either *N*-sulfated or *N*-acetylated and can contain an *O*-sulfate ester at C-6. The uronic acid exists as either a glucoronic or an iduronic acid epimer and may be *O*-sulfated at C-2. In contrast to heparin chains which are extensively sulfated, HS chains are less

20 extensively modified and the sulfate groups are unevenly distributed over the chains due to incompleteness of modification in each step of HS biosynthesis (Lindahl *et al.* 1994) Consequently, HS and heparin are similar due to highly sulfated, heparin-like stretches found in HS.

25 In general, micro-heterogeneity within the structure of GAGs modulates their binding and biological activities (Casu 1991). Their binding properties are usually associated with their anionic sulfate and carboxyl groups. Increasing charge density and molecular weight is often associated with stronger protein binding, but this is not a strict rule (Ruoslahti 1989). Thus, a general increase in binding properties can be achieved by 30 chemical oversulfation (Casu 1991). Most GAGs express their biological properties

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through interactions with plasma and tissue components (Jackson *et al.* 1991; Kjellén *et al.* 1991) and their ubiquitous distribution on cell surfaces make them well suited for microbial attachment.

5 In a particular embodiment, the receptor carbohydrate, or said functional equivalent thereof, is more specifically capable of essentially specific binding to at least one of the segments denoted binding sites of the amino acid sequence according to SEQ ID NO:1, wherein a novel variant of *Plasmodium falciparum* erythrocyte membrane protein (PfEMP1) is disclosed. Even though it is more fully discussed elsewhere in this application, especially in connection with the ligand polypeptide below, it should be understood that said binding segments are sequences, which, according to the present invention, have been found to bind specifically and strongly to glycosaminoglycan-like moieties on receptor substances, such as the ones present on most cells or the present polypeptide. Thus, in a specific embodiment, the receptor carbohydrate according to the invention is capable of binding to an amino terminal part, and preferably an essential part, of the binding segments indicated in SEQ ID NO:1. Consequently, the protein disclosed in SEQ ID NO:1 may be regarded as a ligand, whereas the carbohydrate defined as above may be regarded as the receptor therefore. Most preferably, the receptor binds to an essential part of said sequence or any sequence having a substantial identity or similarity with SEQ ID NO:1.

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The carbohydrate receptor according to the present invention may be prepared by fractionation from animal cells or by conventional methods, which are well known in the art (see *e.g.* Binkley: *Modern Carbohydrate Chemistry*, Marcel Dekker, New York, 1988, with references; and US patents Nos. 5 308 460 and 5 532 147).

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In another aspect, the present invention relates to a carbohydrate as defined above for use as a medicament as well as the use of said receptor as a medicament. Such a medicament is effective in that it is capable of effectively dissolving the rosettes formed by erythrocytes infected by a malaria parasite, as described more detailed in the

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introductory section of this application. Thereby, a medicament comprising the receptor carbohydrate according to the invention in a suitable form will be effective in dissolving and preventing the occlusion of blood vessels, especially in cerebral malaria. Accordingly, the invention also encompasses the use of said carbohydrate in the manufacture of a medicament against malaria, preferably against *P. falciparum* malaria. Thus, in one embodiment, the invention relates to a pharmaceutical composition comprising a carbohydrate according to the invention in a pharmaceutically or veterinary acceptable carrier. Other additives or excipients which are deemed suitable for the specific application may also be present in such a composition. Further, the present invention also relates to any other substance, which has been achieved by use of parts or all of the novel polypeptide defined in SEQ ID NO:1, or, alternatively, by use of a carbohydrate according to the invention as a lead substance, for use as medicaments, for use in the manufacture of pharmaceutical preparations and pharmaceutical compositions comprising such a substance together with a carrier. Methods for producing such substances, which are functionally equivalent to the present carbohydrates, will be discussed in more detail below.

One more aspect relating to the above defined carbohydrate, or malaria protein receptor substance, is a method of treating a patient suffering from a malaria infection, preferably a *P. falciparum* infection. Such a method comprises administering to the patient of an effective amount of the pharmaceutical composition described in detail elsewhere in the present description.

Another aspect of the present invention is a polypeptide, which is capable of acting as a ligand in relation to the receptor carbohydrate described above. Said ligand polypeptide originates from a malaria erythrocyte membrane protein and may be denoted a novel PfEMP1-variant.

Thus, by single-cell RT-PCR, the present inventor has identified a novel PfEMP1-variant of a rosetting parasite. Clusters of GAG-binding motifs have been identified in

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the sequence, denoted binding sites in the sequence listing below. In addition, it has been shown that the recombinant form of the novel PfEMP1-variant, or ligand polypeptide, according to the invention, adheres to solid-phase heparin, to heparan sulfate on the erythrocyte surface, and disrupts rosettes. Further, naturally formed rosettes also seem to be mediated by binding to the same GAG.

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In an advantageous embodiment, the ligand polypeptide according to the invention comprises at least about 300 amino acids of the amino acid sequence according to SEQ ID NO:1. Preferably, the ligand comprises an amino terminal sequence of said sequence, or an analogue thereof. More preferably, the ligand according to the invention comprises about 400-500 amino acids, and most preferably about 400 amino acids (DBL-1), such as about 423 amino acids, thereof. In addition, the ligand polypeptide according to the invention is capable of essentially specific binding to a negatively charged glycosaminoglycan-like moiety. In the preferred embodiment thereof, the ligand polypeptide according to the invention is capable of binding any receptor carbohydrate according to the invention and described above. In a particular embodiment thereof, the polypeptide according to the invention comprises essentially all of the sequence according to SEQ ID NO:1, wherein the complete amino acid sequence of FCR3S1.2 PfEMP1 is shown. The location of potential GAG binding motifs are shown in pink. Motifs no.4, 5 and 9,10 (aa 221-232 and 533-549, respectively) are seen as a single stretch as they are located next to each other (see methods section for description of identification of GAG-binding motifs). These sequence data are available from GenBank under accession number AF003473.

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The weight of the polypeptide according to the invention will depend on the length of the amino acid sequence as discussed above but may be about 100-300 kDa, preferably about 280 kDa. Further, the present invention also relates to any biologically active fragments of the herein disclosed polypeptides and proteins.

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Another aspect of the invention is a method of preparing a ligand polypeptide as defined herein, which comprises the steps of

- (1) the inserting into an expression vector of a nucleic acid encoding said ligand polypeptide or functionally equivalent analogue thereof;
- 5 (2) the transfection of a host cell capable of expressing said nucleic acid with said expression vector to express said polypeptide; and
- (3) the recovery of the expressed polypeptide and advantageously purification thereof to a biologically pure form. Expression vectors, host cells, process parameters etc. are easily chosen by someone skilled in this area.

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Accordingly, a further aspect of the invention is vectors on host cells useful in the above disclosed method and comprising nucleic acids encoding a polypeptide according to the invention.

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In one particular embodiment of the invention, the polypeptide may be cloned using DNA amplification methods, such as the polymerase chain method (PCR) (see e.g. Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbour, N.Y., 1989; Berger & Kimmel, *Methods in Enzymology*, Vol. 152: *Guide to Molecular Cloning Techniques*, Academic Press, Inc., San Diego, Calif., 1987; Co *et al* 20 (1992) *J. Immunol.* 148:1149). Thus, for example, the nucleic acid sequence, which will be disclosed in more detail below, or subsequence is PCR amplified using a sense primer containing one restriction site and an antisense primer containing another restriction site. This will produce a nucleic acid encoding the desired sequence or subsequence having terminal restriction sites. This nucleic acid can then easily be 25 ligated into a vector having appropriate corresponding restriction sites. Suitable PCR primers are easily chosen by one of skill in the art based on the sequence to be expressed. Appropriate restriction sites can also be added by site-directed mutagenesis (see Gillman & Smith (1979) *Gene*, 8: 81-97; Roberts *et al.* (1987) *Nature* 328: 731-734).

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The nucleic acids according to the invention, which are described in more detail below, may be expressed in a variety of host cells, including *E. coli*, other bacterial hosts, yeast and various higher eucaryotic cells, such as the COS, CHO and HeLa cell lines and myeloma cell lines. The recombinant protein gene will be operably linked to appropriate expression control sequences for each host. For *E. coli*, this includes a promoter, such as the T7, trp, or lambda promoters, a ribosome binding site and preferably a transcription termination signal. For eucaryotic cells, the control sequences will include a promoter and preferably an enhancer derived from immunoglobulin genes, SV40, cytomegalovirus *etc.*, and a polyadenylation sequence, and may include splice donor and acceptor sequences. The plasmids can be transferred into the chosen host cell by well-known methods, such as calcium chloride transformation for *E. coli* and calcium phosphate treatment or electroporation for mammalian cells. Cells transformed by the plasmids can be selected by resistance to antibiotics conferred by genes contained on the plasmids, such as amp, gpt, neo and hyg genes.

Once expressed, the recombinant polypeptides according to the invention may be purified according to standard procedures of the art, including ammonium sulfate precipitation, affinity columns, column chromatography, gel electrophoresis and the like (see generally R. Scopes, *Protein Purification*, Springer-Verlag, N.Y. (1982), Deutcher, *Methods in Enzymology* vol. 182: *Guide to Protein Purification*, Academic Press, Inc. N.Y. (1990)). Substantially pure compositions are preferred, such as at least about 90-95% homogeneity, and most preferred, 98-99% homogeneity. Once purified, partially or to the homogeneity as desired, the polypeptides according to the invention may be used, *e.g.* as immunogens for antibody production.

As one of skill in this field would recognize, after chemical synthesis, biological expression, or purification, the proteins, polypeptides or fusion proteins according to the present invention may possess a conformation substantially different from the native conformations of the constituent polypeptides. In this case, it may be necessary

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to denature and reduce the polypeptide and then to cause the polypeptide to re-fold into the preferred conformation. Methods of reducing and denaturing proteins and inducing re-folding are well known to those of skill in the art (see e.g. Debinski *et al.* (1993) *J. Biol. Chem.*, 268: 14065-14070; Kreitman and Pastan (1993) *Bioconj. Chem.*, 4:581-585; and Buchner *et al.* (1992) *Anal. Biochem.*, 205:263-270).

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One of skill would recognize that modifications can be made to the present polypeptides and proteins without diminishing their biological activity, e.g. in order to facilitate cloning, expression or incorporation of the targeting molecule into a fusion protein. Such modifications are well known and any thus modified peptides and proteins are understood to be encompassed within the scope of the present invention as defined by the appended claims.

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Further, the present invention also relates to vectors and recombinant host cells comprising the sequence according to SEQ ID NO:1 or any biologically active fragment thereof. In addition, the invention also encompasses any isolated cell comprising the sequence according to SEQ ID NO:1 as an endogenous gene, which has been manipulated by gene activation, that is, wherein additional regulatory sequences have been introduced in order to increase the expression of the native coding sequence (see e.g. US patent no. 5 578 461 and US patent no. 5 641 670). The culture of cells according to the present invention is well known in the art (see e.g. Freshney, *Culture of Animal Cells, A Manual of Basic Technique*, third ed., Wiley-Liss, New York (1994) and the references cited therin for a general guide to the culture of cells).

As indicated above, the present invention also relates to nucleic acids encoding the polypeptides according to the invention as well as to any nucleic acid capable of specific hybridization, under stringent conditions, to such a nucleic acid. The nucleic acids according to the invention thus include, but are not limited to, DNAs, RNAs, an mRNA transcript, a cDNA reverse transcribed from a mRNA, and RNA transcribed from that cDNA, a DNA amplified from the cDNA, an RNA transcribed from the

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amplified DNA or subsequences of any of these nucleic acids and may be prepared by any suitable method, which is easily chosen by someone skilled in this field. Such methods include, for example, cloning and restriction of appropriate sequences or direct chemical synthesis by methods such as the phosphotriester method (Narang *et al.*, *Meth. Enzymol.* 68:90-99 (1979)); the phosphodiester method (Brown *et al.*, *Meth. Enzymol.* 68: 109-151 (1979)); the diethylphosphoramide method (Beaucage *et al.*, *Tetra. Lett.*, 22:1859-1862 (1981)); and the solid support method (US Patent no. 4 458 066).

Chemical synthesis produces a single stranded oligonucleotide. This may be converted into double stranded DNA by hybridization with a complementary sequence or by polymerisation with a DNA polymerase using the single strand as a template. The DNA sequence obtained by chemical synthesis is limited in length by the technique used, however, longer sequences are easily obtained by the ligation of such shorter sequences to the desired length. Alternatively, subsequences may be cloned and the appropriate subsequences cleaved using suitable restriction enzymes. The fragments may then be ligated to produce DNA sequences of the desired size.

A nucleic acid according to the invention may *e.g.* be used in the above method for preparing a polypeptide, as a probe *etc.* For the use thereof as a probe, it is often desirable to label the sequence with a detectable label. The label may be incorporated during the amplification step in the preparation of the nucleic acid, *e.g.* by PCR, by transcription amplification using a labelled nucleotide incorporating a label in the transcribed nucleic acid or, alternatively, by direct addition thereof, such as by nick-translation or any other suitable method. Detectable labels suitable for use according to the present invention include any composition detectable by spectroscopic, photochemical, biochemical, immunochemical, electrical, optical or chemical means and methods for the detection thereof are well known to those of skill in the art.

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In an alternative aspect of the invention, the polypeptide is produced in the form of a recombinant fusion protein, which comprises said polypeptide, comprising suitable glycosaminoglycan-like moieties, fused to another protein or polypeptide. Such a polypeptide comprising fusion protein may be more advantageous for certain applications than the polypeptide *per se*. In a preferred embodiment of the invention, the fusion proteins are synthesized using recombinant nucleic acid methodology. Generally, this involves creating a nucleic acid that encodes the receptor-targeted fusion molecule, placing the nucleic acid in an expression cassette under the control of a suitable promoter, expressing the protein in a host, isolating the expressed protein and, if required, renaturing the protein. Techniques sufficient to guide one of skill through such procedures are found in *e.g.*, Sambrook *et al.*, *Molecular Cloning. A Laboratory Manual*, 2nd ed., vol. 1-3, Cold Spring Harbor Laboratory, 1989; Berger and Kimmel, *Guide to Molecular Cloning Techniques*, Methods in Enzymology, vol. 152, Academic Press, Inc., San Diego, CA; and *Current Protocols in Molecular Biology*, F.M. Ausubel *et al.*, eds., Current Protocols (Greene Publishing Associates, Inc. And John Wiley & Sons, Inc.) (1994 Supplement). Alternatively, the fusion protein is produced by any method for chemical synthesis well known in the art. Thus, a fusion protein according to the invention may comprise any polypeptide, antibody or biologically active fragment thereof according to the present invention. The effects of exemplary fusion proteins on rosetting is *e.g.* discussed in the Experimental section of this specification, see *e.g.* Fig 4.

The present invention also relates to a polypeptide according to the invention for use as a medicament as well as the use thereof in the manufacture of a medicament. Such a medicament is effective in that it is capable of effectively dissolving the rosettes formed by erythrocytes infected by a malaria parasite, as described more detailed in the introductory section of this application. Thereby, a medicament comprising the polypeptide according to the invention, *per se* or in the form of a fusion protein, will be effective in dissolving and preventing the occlusion of blood vessels, especially in cerebral malaria. In addition, the above described rosettes have also been shown to be

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associated with other severe complications of malaria (Carlson, J., et al, Lancet 336, 1457-1460 (1990); Treutiger et al, Am. J. Trop. Med. Hyg. 46, 503-510 (1992); and Rowe, A et al, Inf. Immun 63, 2323-2326 (1995)). Thus, a medicament comprising a polypeptide according to the invention is also effective in the treatment of such other conditions. Accordingly, the invention also encompasses the use of said polypeptide in the manufacture of a medicament against malaria, preferably *P. falciparum* malaria, and/or other severe complications of malaria. Consequently, in a further aspect, the invention relates to a pharmaceutical preparation comprising said polypeptide or fusion protein in a pharmaceutically or veterinary acceptable carrier, e.g. an aqueous carrier, such as buffered saline or the like. These solutions are sterile and generally free of any undesirable matter. They may be sterilized by conventional, well known sterilization techniques. The pharmaceutical preparations according to the invention may be used in any suitable method of administration, such as in parenteral, topical, oral or even local administration for prophylactic and/or therapeutic treatment and may be administered in a variety of unit dosage forms depending on the administration method chosen. Further, the pharmaceutical compositions according to the invention may comprise any pharmaceutically acceptable auxiliary substances required to approximate physiological conditions, such as pH adjusting and buffering agents, toxicity adjusting agents and the like, for example sodium acetate, sodium chloride, potassium chloride, calcium chloride, sodium lactate etc. Actual methods for preparing the appropriate administrable compositions will be known or apparent to those skilled in this field and are described in more detail in e.g. *Remington's Pharmaceutical Science*, 15th ed., Mack Publishing Company, Easton, Pennsylvania (1980). (For a brief review of methods for drug delivery, see Langer, Science 249:1527-1533 1990)).

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Accordingly, another aspect of the present invention is a method of treating a patient suffering from a malaria infection by the administration of the present polypeptide or a fusion protein or, alternatively, preventing malaria in a subject at risk of being infected thereof. It is to be understood, that in the present context, the term "preventing" refers to the prevention of a disorder, such as malaria, or the symptoms thereof, by use of a

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medicament as well as such a prevention by use of a vaccine composition. Thus, the present invention encompass preventive or prophylactic treatments and medicaments used therein, which treatments will include drugs that actively participate in the prevention of a particular disorder, such as malaria or the symptoms thereof. Further, 5 the invention also encompasses treatments that elicit a preventive response from a patient, such as an immunological response in the case of vaccination. Consequently, "a medicament for the prevention of malaria" includes also includes vaccine compositions. The method comprises administering to said patient of an effective amount of the herein described pharmaceutical composition, said amount being dependent on the severity of the disease and the general state of the health of the patient. Preferably, the malaria infection treated by the method according to the invention is a *P. falciparum* infection.

In the preferred embodiment, the vaccine compositions according to the invention will include a molecule according to the invention, such as a polypeptide, in an immunologically effective amount together with a suitable pharmaceutically acceptable carrier. In this context, examples of suitable carriers are e.g. thyroglobulin, albumins, such as human serum albumin, tetanus toxoid, polyamino acids, such as poly(D-lysine; D-glutamic acid), influenza, hepatitis B virus core protein as well as other carriers well known to those skilled in the art. The vaccines may also include a physiologically tolerable diluent, such as water, buffered water, buffered saline, saline and they may further also include any suitable adjuvant, such as incomplete Freunds' adjuvant, aluminium phosphate, aluminium hydroxide, alum, ammonium hydroxide in case of human patient, etc. The immune response of the patient may include generation of 20 antibodies, activation of cytotoxic T-lymphocytes against cells expressing the polypeptides or other mechanisms known within this field. (See e.g. Paul, *Fundamental Immunology*, 2nd ed., Raven Press; Sedagh et al., *Proc. Natl. Acad. Sci.* (1994) 91:9866-9877; US patent no. 4 722 848; and Langford, C.L. et al., 1986, *Mol. Cell. Biol.* 6:3191-3199.)

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Another highly interesting and advantageous aspect of the present invention is the use of the polypeptide as defined above as a model substance for identifying substances binding to a malaria erythrocyte membrane protein, such as the novel variant disclosed in SEQ ID NO:1, or analogues thereof. Thereby, new receptor substances effective in the treatment and/or prevention of malaria infections may be isolated from suitable environments or bulks comprising the same.

More specifically, a polypeptide according to the invention, or a fragment thereof, is used in the design of an organic compound, which is modeled to resemble the three dimensional structure of the amino acid residues of said polypeptide. Such a design of mimetics to a known pharmaceutically active compound is a known approach to the development of pharmaceuticals based on a "lead" compound. Mimetic design, synthesis and testing is generally used to avoid randomly screening large number of molecules for a target property. In relation to mimetics, or mimics, as they are sometimes denoted, see for example Shikman A.R. and Cunningham M.W., *J. Immunol.* 152(9):4375 (1994); Vaughan *et al.*, *Xenotransplantation* 3:18-23 (1996); and Koogman *et al.*, Dean (1994), *BioEssays*, 683-687; Cohen and Shatzmiller (1993), *J. Mol. Graph.*, 11: 166-173; Wiley and Rich (1993), *Med. Res. Rev.*, 13:327-384; Moore (1994), *Trends Pharmacol. Sci.*, 15:124-129; Hruby (1993), *Biopolymers*, 33: 1073-1082; Bugg *et al.* (1993), *Sci. Am.*, 269:92-98.

In brief, the particular parts of the compound that are critical and/or important are firstly determined. This can be done systematically by varying the amino acid residues in the peptide, e.g. by substituting each residue in turn. These parts or residues constituting the active region of the compound are known as its "pharmacophore". Once the pharmacophore has been found, its structure is modelled according to its physical properties, e.g. stereochemistry, bonding, size and/or charge, using data from a range of sources, e.g. spectroscopic techniques, X-ray diffraction data and NMR. Computational analysis, similarity mapping (which models the charge and/or volume

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of a pharmacophore, rather than the bonding between atoms) and other techniques can be used in this modelling process.

In a specific embodiment, the three dimensional structure of the ligand and its binding partner are modelled. This can be especially useful where the ligand and/or binding partner (receptor) change conformation on binding, allowing the model to take account of this in the design of the mimetic.

A template molecule is then selected onto which chemical groups which mimic the pharmacophore can be grafted. The template molecule and the chemical groups grafted onto it can conveniently be selected so that the mimetic is easy to synthesize, is likely to be pharmacologically acceptable and does not degrade *in vivo*, while retaining the biological activity of the lead compound. The mimetic(s) found by this approach can then be screened to see whether or not they have the target property, or to what extent they exhibit it. Further optimisation or modification can then be carried out to arrive at one or more final mimetics for *in vivo* or clinical testing. Accordingly, the use of the present polypeptide ligand and/or carbohydrate binding partner as models to identify optimal pharmaceutically active substances, preferably for preventing and/or treating malaria, is encompassed by the present invention as are any substances identified thereby, which fulfills the herein desired criteria.

In another aspect, the invention relates to diagnostic and screening assays, wherein *e.g.* the polypeptides according to the invention are used for the detection of antibodies or wherein antibodies as disclosed below are used to detect PfEMP1 or fragments thereof. Further, nucleic acids may be detected in biological samples. (For a review of diagnostic immunoassay procedures, see *e.g.* *Basic and Clinical Immunology*, 7th ed., Stites, D., and Terr, A., 1991.)

One further aspect of the invention is an antibody, which is specifically immunoreactive with a ligand polypeptide according to the present invention. Such an

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antibody may be used to formulate another pharmaceutical composition together with suitable pharmaceutically and/or veterinary acceptable carriers, excipients etc.

Accordingly, the present invention also relates to such antibody compositions as well as to methods of treating and/or preventing malaria infection in a patient. Such

5 methods comprises administering to said patient of an effective amount of the pharmaceutical composition defined above. Most preferably, the infection to be treated and/or prevented is a *P. falciparum* infection.

10 Thus, antibodies are raised to the polypeptides of the present invention, including individual, allelic, strain, or species variants, and fragments thereof, both in their naturally occurring (full-length) forms and in recombinant forms. Additionally, antibodies are raised to these polypeptides in either their native configurations or in non-native configurations. Anti-idiotypic antibodies can also be generated. Many methods of making antibodies are known to persons of skill. (In this context, see e.g. *Fundamental Immunology*, Third Edition, W.E. Paul, ed., Raven Press, N.Y. 1993.) While various antibody fragments are defined in terms of the digestion of an intact antibody, one of skill will appreciate that such fragments may be synthesized *de novo* either chemically or by utilizing recombinant DNA methodology. Thus, the term antibody, as used herein, also includes antibody fragments either produced by the modification of whole antibodies or 20 those synthesized *de novo* using recombinant DNA methodologies (e.g., single chain Fv).

25 A number of immunogens are used to produce antibodies specifically reactive with polypeptides according to the invention. Recombinant or synthetic polypeptides according to the invention of 8-15, preferably 10, amino acids in length, or greater, are the preferred polypeptide immunogen (antigen) for the production of monoclonal or polyclonal antibodies.

30 Methods of producing polyclonal antibodies are known to those of skill in the art. See, e.g., Coligan (1991) *Current Protocols in Immunology* Wiley/Greene, NY; and Harlow and Lane (1989) *Antibodies: A Laboratory Manual* Cold Spring Harbor Press, NY.

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Monoclonal antibodies are prepared from cells secreting the desired antibody. These antibodies are screened for binding to normal or modified polypeptides, or screened for agonistic or antagonistic activity, *e.g.*, activity mediated through a *suppressor of fused* 5 protein. Specific monoclonal and polyclonal antibodies will usually bind with a K_D of at least about 0.1 mM, more usually at least about 50 μ M, and most preferably at least about 1 μ M or better.

In some instances, it is desirable to prepare monoclonal antibodies from various mammalian hosts, such as mice, rodents, primates, humans, *etc.* Description of techniques for preparing such monoclonal antibodies are found in, *e.g.* Stites *et al.* (eds) *Basic and Clinical Immunology* (4th ed.) Lange Medical Publications, Los Altos, CA, and references cited therin; Harlow and Lane, *supra*; Goding (1986) *Monoclonal Antibodies: Principles and Practice* (2nd ed.) Academic Press, New York, NY; and Kohler and Milstein (1975) *Nature* 256: 495-497.

Other suitable techniques involve selection of libraries of recombinant antibodies in phage or similar vectors (*see, e.g.*, Huse *et al.* (1989) *Science* 246: 1275-1281; and Ward, *et al.* (1989) *Nature* 341: 544-546; and Vaughan *et al.* (1996) *Nature Biotechnology*, 14: 20 309-314).

Advantageously, the polypeptides and antibodies according to the invention are labelled by joining, either covalently or non-covalently, a substance which provides for a detectable signal. A wide variety of labels, and conjugation techniques are known and are reported 25 extensively in both the scientific and patent literature. See for example patents teaching the use of such labels, including U.S. Patent Nos. 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149; and 4,366,241.

In a specific embodiment of the invention, the present antibodies are used for affinity 30 chromatography in isolating polypeptides. Columns are prepared, *e.g.*, with the antibodies

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linked to a solid support, *e.g.*, particles such as agarose, Sephadex, or the like, where a cell lysate is passed through the column, washed, and treated with increasing concentrations of a mild denaturant, whereby purified polypeptides are released.

5 The antibodies can be used to screen expression libraries for particular expression products. Usually the antibodies in such a procedure are labelled with a moiety allowing easy detection of presence of antigen by antibody binding.

10 Antibodies raised against the present polypeptides can also be used to raise antiidiotypic antibodies. These are useful for detecting or diagnosing the various pathological conditions related to the presence of the respective antigens.

15 The antibodies of this invention can also be administered to an organism (*e.g.*, a human patient) for therapeutic purposes. A large number of methods of generating chimeric antibodies are well known to those of skill in the art (*see, e.g.*, U.S. Patent Nos: 5,502,167, 5,500,362, 4,491,088, 5,482,856, 5,472,693, 5,354,847, 5,292,867, 5,231,026, 5,204,244, 5,202,238, 5,169,939, 5,081,235, 5,075,431, and 4,975,369). For references regarding human antibodies, *see, e.g.*, Larrick *et al.*, U.S. Pat. No 5,001,065, for review).

20 Human antibodies of the present invention may *e.g.* be produced initially in trioma cells. The general approach for producing human antibodies by trioma technology has been described by Ostberg *et al.* (1983) *Hybridoma* 2: 361-367, Ostberg, U.S. Pat. No. 4,634,664, and Engelman *et al.* U.S. Pat. No. 4,634,666.

25 As someone skilled in this field easily realises, the receptor carbohydrates, the ligand polypeptide and the antibodies according to the invention are also useful for a wide variety of diagnostic purposes, such as in immuno assays. Methods, other reagents, amounts *etc* are easily determined by someone skilled in the area on the basis of the information given in this application (for immuno assays, *see e.g.* Harlow and Lane

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(1988) *Antibodies, A Laboratory Manual*, Cold Spring Harbour Publications, New York).

Detailed description of the drawings

5 In addition to the description given in this section, the drawings will also be commented under "Results" below.

Figure 1. Identification of rosetting PfEMP1.

10 Figure 1A rosetting, single *P. falciparum*-infected erythrocyte is seen by light microscopy held by a 5 µm micro-pipette (A, 1). The uninfected erythrocytes are stripped of the infected cell and careful examination confirms that it indeed is infected by a single parasite (A, 2-3).

15 Figure 1B shows the amplification of a 434-bp band in 4 (from reaction 3, 4, 5 and 7) out of 8 single-infected, rosetting erythrocytes using degenerate primers generated from the primary sequence of the DBL-1 domain of PfEMP1.

Figure 1C shows the amplification pattern with the same primers as in B of bulk cultures of rosetting (R⁺) FCR3S1.2 cultures and the R⁻ FCR3S/a parasites. Note that the 434-bp product is only seen with the R⁺ parasites.

20 Figure 1D shows the hybridisation pattern in Northern blotting of the 434-bp sequence to mRNA extracted from the highly rosetting parasite FCR3S1.2 (84% R⁺) and the weak hybridisation to the R⁻ FCR3S/a parasite (9% R⁺).

25 Figure 1E shows the autoradiograph of a Triton-X 100 insoluble, SDS-soluble extract of FCR3S1.2 infected erythrocytes after radio-iodination labelling. PfEMP1 (arrowed) is labelled on FCR3S1.2 infected erythrocytes and is cleaved by low concentrations of trypsin.

Figure 2. Map of cDNA structure, sequencing clones, deduced amino acid sequence and the location of GAG binding motifs in the rosetting PfEMP1 of FCR3S1.2. A shows the location of 434-bp fragment and the three fragments (I, II and III) which were initially cloned for sequencing. Restriction enzyme-digestion sites are indicated by

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arrows. Additional overlapping clones used for sequencing are shown below. **B** shows the primary structure of the rosetting FCR3S1.2-PfEMP1. It has two Duffy binding-like (DBL) domains (DBL-1 and 4), one cysteine-rich interdomain region (CIDR), one transmembrane (TM) region and one acidic C-terminal segment (ATS). **C** shows the distribution of amino acids in different regions of FCR3S1.2-PfEMP1.

Figure 3. Rosetting FCR3S1.2-PfEMP1 binds to heparan sulfate. All the gels are 10% SDS-PAGE stained with Coomassie. **A** shows the expressed GST, DBL-1-GST or ATS-GST after purification on glutathione-sepharose and SDS-PAGE. **B** shows the binding capacity of different fusion proteins to heparin-sepharose after SDS-PAGE. **C** shows the inhibition produced by different glycosaminoglycans on the binding of DBL-1-GST to heparin-sepharose followed by SDS-PAGE. **D & E** show the binding of DBL-1-GST (**D**) and ATS-GST (**E**) to monolayers of normal RBC as visualised by a mAb to GST labelled with biotin and FITC-avidin.

Figure 4. Disruption of pre-formed, natural rosettes with either DBL-1-GST or ATS-GST.

Results are means and standard errors of three experiments.

Figure 5. Effect of glycosaminoglycans (GAGs) on *P. falciparum* rosetting. **A** shows disruption of rosettes exerted by different GAGs. FCR3S1.2 cultures were incubated with GAGs for 1 h at 37° C and compared to control culture. Results are the means and standard error of three separate experiments. **B** shows the effect of enzyme treatment of uninfected, C-FDA labelled, erythrocytes in a competitive assay of rosette-reformation in the presence of normal erythrocytes and FCR3S1.2 infected pRBC. Results are the means and standard error of three separate experiments; two experiments for neuraminidase.

EXPERIMENTAL

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The following examples are only intended to illustrate the present invention and do not limit the scope of the invention as defined by the appended claims. Throughout the present application, the following abbreviations are used:

PfEMP 1, *Plasmodium falciparum* erythrocyte membrane protein 1; DBL, Duffy binding-like; GST, glutathione S-transferase; GAG, glycosaminoglycan; pRBC, parasitised RBC; ICAM-1, intercellular adhesion molecule 1 ; TM, transmembrane.

EXAMPLE 1

10 **Materials and Methods**

The Parasites

The *P. falciparum* parasites were cultured according to standard methods with 10% AB⁺ Rh⁺ serum added to the buffered medium (RPMI supplemented with hepes, gentamycin and sodium bicarbonate). The FCR3S1.2 was cloned with micro-manipulation from the previously limiting-dilution cloned parasite FCR3 (Udomsangpetch, R., B. Wåhlin, J. Carlson, K. Berzins, M. Torii, M. Aikawa, P. Perlmann, and M. Wahlgren. 1989. *Plasmodium falciparum*-infected erythrocytes form spontaneous erythrocyte rosettes. *J. Exp. Med.* 169, 1835-1840). Its rosetting rate was routinely >80% (R⁺). FCR3S/a was negatively enriched for low rosetting from FCR3 using Ficoll-isopaque (<10%, R⁻). It should be noted that FCR3S was previously called Palo Alto (Uganda) in our publications (Carlson, J., H.P. Ekre, H. Helmby, J. Gysin, B.M. Greenwood, and M. Wahlgren. 1992. Disruption of *Plasmodium falciparum* erythrocyte rosettes by standard heparin and heparin devoid of anticoagulant activity. *Am. J. Trop. Med. Hyg.* 46, 595-602, Udomsangpetch, R., B. Wåhlin, J. Carlson, K. Berzins, M. Torii, M. Aikawa, P. Perlmann, and M. Wahlgren. 1989. *Plasmodium falciparum*-infected erythrocytes form spontaneous erythrocyte rosettes. *J. Exp. Med.* 169, 1835-1840, Scholander, C., C.J. Treutiger, K. Hultenby, and M. Wahlgren. 1996. Novel fibrillar structure confers adhesive property to malaria-infected erythrocytes. *Nature Med.* 2, 204-208, Carlson, J. and M. Wahlgren, 1992. *Plasmodium falciparum* erythrocytes rosetting is mediated by promiscuous lectin-like interactions. *J. Exp. Med.*

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176, 1311-1317, Carlson, J., G. Holmquist, D.W. Taylor, P. Perlmann, and M. Wahlgren. 1990. Antibodies to a histidine-rich protein (PfHRP1) disrupt spontaneously formed *Plasmodium falciparum* erythrocyte rosettes. *Proc. Natl. Acad. Sci. U.S.A* 87, 2511-2515, Helmby, H., L. Cavelier, U. Pettersson, and M. Wahlgren. 1993. Rosetting 5 *Plasmodium falciparum*-infected erythrocytes express unique antigens on their surface. *Infect. Immun.* 61, 284-288). Molecular studies of the "Palo Alto" parasites have revealed, however, that they are identical to parasites of the FCR3 lineage (Fandeur, T., S., O. Bonnefoy, Mercereau-Puijalon. 1991. In vivo and in vitro derived Palo Alto 10 lines of *Plasmodium falciparum* are genetically unrelated. *Mol. Biochem. Parasitol.* 47, 167-178).

Optimisation of single *P. falciparum* RT-PCR

Two degenerate primers (DBL-1.1, 5'-GG(A/T) GC(A/T) TG(TC) GC(A/T) CC(A/T) T(A/T)(T/C) (A/C)G -3'; DBL-1.2, 5'-A(A/G)(A/G)T A(T/C)TG (T/A)GG (A/T)AC (A/G)TA (A/G)TC -3' which mapped to the conserved region of all PfEMP1 DBL-1 15 were modified from the sequences of Su *et al.* The amplification parameters were first optimised so that the amplified products were visible with normal ethidium bromide (EB) staining (Cobb, B. D. and J.M. Clarkson, 1994. A simple procedure for optimising the polymerase chain reaction (PCR) using modified Taguchi methods.

20 *Nucl. Acid. Res.* 22, 3801-3805). Briefly, one to five parasites, obtained by limiting dilution, were directly emerged in the RT-PCR buffer (Stratagene) with different concentration of primers, MgCl₂, KCl and Tris-Cl. Both DNA and RNA were released from the parasites by heating at 93°C for 3 min. The DNA was degraded by addition of 10 U DNase (Stratagene). Reverse transcription was carried out immediately after 25 addition of random primers and reverse transcriptase (Perkin-Elmer). The PCR reaction was subsequently performed in the same tube. Through comparison of the amplification efficiency from different reactions, the optimised parameters for single cell RT-PCR were found to be as follows: 100mM Tris-Cl, pH 8.3, 35mM MgCl₂, 500mM KCl, and the final concentration of primers was 1 μM. In the subsequent 30 experiments, individual trophozoite-infected rosetting erythrocytes were isolated with

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a 5 μ m glass-pipette using an inverted microscope. The selected pRBC was stripped of uninfected RBC and repeatedly grabbed, ejected and turned to conclusively ensure that it had pigment and that the selected cell was a single trophozoite-infected RBC (see Fig. 1A). Fifty cycles of amplification at 93°C for 20 seconds, 55°C for 30 seconds and 72°C for 1 min were needed for product detection. Several controls were included in each experiment; one blank control (without parasite(s)) and one without reverse-transcriptase to rule out the possibility of contamination and amplification due to the presence of genomic DNA.

10 RT-PCR with total RNA from bulk-cultured FCR3S1.2 or FCR3S/a and Northern-blot analysis

Total RNA purification from both FCR3S1.2 (R⁺) and FCR3S/a (R⁻) parasite and RT-PCR was performed as described (Sambrook, J., E.F., Fritsch, and T., Maniatis. 1989. Molecular Cloning, A laboratory manual, second edition. Cold Spring Harbor Laboratory Press). All the amplified products were cloned with Original TA Cloning Kit (Invitrogen) and sequenced. Northern blot analysis was carried out using standard methods (Sambrook, J., E.F., Fritsch, and T., Maniatis. 1989. Molecular Cloning, A laboratory manual, second edition. Cold Spring Harbor Laboratory Press). Membranes were probed overnight at 60°C using the 434-bp fragment labelled with a-³²P-dCTP. Washing was performed under stringent conditions (60°C, 0.1xSSC) and the blots were examined in a Molecular Dynamics phosphoimager.

Cloning and Sequencing of the whole cDNA

25 A specific upstream primer (L-6, 5'-GAC ATG CAG CAA GGA GCT TGA TAA -3') in the 434-bp sequence and a downstream primer (L-5, 5'-CCA TCT CTT CAT ATT CAC TTT CTG A -3') mapping to the conserved sequence of ATS were generated and reverse transcription was carried out as described above. PCR was performed with the Expand™ High Fidelity PCR System (Boehringer Mannheim). A single 4.9-kb fragment was amplified, which was digested into three fragments with Hind III and 30 EcoR V and cloned into the pZErO-1 vector (Zero Background, Invitrogen). The

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sequencing was performed with LongRanger™ gel (FMC) on an A.L.F. Sequencer (Pharmacia). The 5' region of the FCR3S1.2-*var1* transcript was cloned by screening a cDNA library (Schlichtherle, unpublished) with the 434-bp fragment as probe and seven overlapping fragments were sequenced. The 3' terminal region was cloned by nested RT-PCR. Reverse transcription was primed with oligo-dT and PCR was performed with a specific 5' primer (P-1, 5'-CTT TCG ACT CTA CCA TCC T -3') upstream of TM region and a 3' primer (P-4, 5'-TTA GAT ATT CCA TAT ATC TGA TA -3') mapping to the C-terminal sequence of FCR3 (*var 2*) PfEMP 1. Five overlapping fragments were sequenced. Fourteen overlapping clones were in total sequenced in both directions in order to ensure that the sequence was correct and was transcribed from a single gene.

Sequence analysis

The DNA and amino acid sequence analysis (editing, translation, peptidesort, plot, etc.) was performed with the Genetic Computer Group (GCG) (Devereux, J., P. Haeblerli, and O. Smithies. 1984 A comprehensive set of sequence analysis programs for VAX. *Nucl. Acid. Res.* 12, 387-395) program. Alignment of the deduced amino acid sequence with the published PfEMP1 sequences in the GenBank was made to identify the sequence. The identification of potential GAG-binding motifs in FCR3S1.2-*var1* and in the other published PfEMP1 sequences was done by firstly searching for the presence's of either of the 4 amino-acid motifs KK, KR, RK, or RR, and secondly manually checking each of the identified sequences for the presence of the motifs XBBXBX or XBBBXXBX where B is a basic amino acid (K,R,H) and X is a hydrophobic residue. A certain degree of liberty concerning the location of the basic residues in the motifs is acceptable (Cardin, A. D. and H.J.R. Weintraub, 1989. Molecular modeling of protein-glycosaminoglycan interactions. *Arteriosclerosis* 9, 21-32).

Expression of DBL-1 or ATS using the pGEX-4T-1 vector

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Both DBL-1 and ATS fragments were amplified by specific primers (Ex-1.1, 5'-ATC GAA TTC TGC AAA AAA GAT GGA AAA GGA A -3' and D-1, 5'-GTA TTT TTT TTG TTT GTC AAA TTG -3' for DBL-1; Ex-2, 5'-ATC GAA TTC TCT GAA AAT TTA TTC CAA A -3' and P-4 for ATS). The amplified fragments were inserted into the EcoR I cloning site of pGEX-4T-1 downstream of the glutathione S-transferase sequence. The *E.coli* BL21 was used as the expression strain. Expression of both fusion proteins was induced with 0.1mM IPTG at 30°C for 4h and the fusion proteins were purified on glutathione sepharose (Pharmacia) as described in the instructions provided by the manufacturer (GST Gene Fusion System, Pharmacia). The expression constructs were sequenced by cycle sequencing to check that the recombinant plasmids were of the expected sequences in the correct reading frames. Thrombin cleavage of the fusion proteins was performed according to a standard procedure. Western-blot analysis of DBL-1-GST and ATS-GST fusion proteins was with a biotin labelled anti-GST mAb (clone GST-2, IgG2b, Sigma) and ALP-avidin (Sigma) to reveal the pattern of protein expression. Although the induction of expression was at a low temperature and the purification was in the presence of a cocktail of enzyme inhibitors (0.5mM EDTA, 1mM Pefabloc®SC (AEBSF), Boehringer Mannheim), there was still some breakdown of the DBL-1-GST. The fusion proteins, stained by the anti-GST mAb, decreased with thrombin treatment. This information together with the knowledge that the plasmids were of the expected sequences ensured that the fusion proteins indeed were the correct ones.

Heparin binding and blocking assay

The potential binding of the fusion proteins to heparin was studied by mixing either DBL-1-GST, ATS-GST fusion protein or GST alone (20 µl, 150 µg/ml in PBS) with 20 µl of 50% heparin-sepharose (Pharmacia) and incubating for 5 min at room temperature in the absence of serum; binding to un-coupled sepharose was used as control. The heparin-sepharose and protein mixture was washed 3 times in large volumes of PBST buffer (PBS plus 0.05% Tween-20) before extracting bound proteins in loading-buffer for 10% SDS-PAGE. Inhibition of DBL-1-GST fusion protein

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binding to heparin with heparin, heparan sulfate or chondroitin sulfate (Lövens Kemiske Fabrik, Denmark, more than 90% pure) was also tested. Twenty μ l of 150 μ g/ml DBL-1-GST fusion protein was mixed separately for 5 minutes with an equal volume of heparin, heparan sulfate, or chondroitin sulfate, titrated from 10 to 0.5 mg/ml, before the addition of heparin sepharose. The inhibitory activities were checked by SDS-PAGE.

Erythrocyte binding and blocking assay

Ten-well immunofluorescence glass-slides were precoated with 10% poly-L-lysine in PBS for 30 min. Monolayers of RBC were made by addition of 20 μ l of 0.5% 3x washed bloodgroup O Rh⁺RBC in PBS to each well. Twenty μ l DBL-1-GST, ATS-GST or GST alone (80 μ g/ml) in PBS was added to the wells for 30 min. The DBL-1-GST fusion protein was in subsequent experiments incubated in the presence of heparin, heparan sulfate or chondroitin sulfate (titrated from 20 to 8 mg/ml) to study the inhibitory activity of each GAG. Slides were washed 3 times with PBS and the fusion protein-binding was detected with the biotin-labelled anti-GST mAb and an ExtraAvidin[®]FITC conjugate (Sigma). The fluorescence was assessed in a Nikon Optiphot-2 UV microscope, using a $\times 10$ ocular and an oil lens with a magnification of $\times 100$.

Rosette disruption assay

The assay was performed essentially as described (Carlson, J. and M. Wahlgren, 1992. *Plasmodium falciparum* erythrocytes rosetting is mediated by promiscuous lectin-like interactions. *J. Exp. Med.* 176, 1311-1317). The recombinant fusion proteins (25 μ l in PBS of DBL-1-GST or ATS-GST) were added to 25 μ l aliquots of a \approx 80% rosetting culture of FCR3S1.2 in a micro-titre plate. The mixtures were incubated for 30 min. at 37°C after which the rosetting rate was scored and compared to mock treated controls after staining with acridine orange.

Enzyme treatment of normal RBC

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Human bloodgroup O Rh⁺ erythrocytes (5% suspension in RPMI) were, previous to C-FDA labelling (Carlson, J., G. Holmquist, D.W. Taylor, P. Perlmann, and M. Wahlgren. 1990. Antibodies to a histidine-rich protein (PfHRP1) disrupt spontaneously formed *Plasmodium falciparum* erythrocyte rosettes. *Proc. Natl. Acad. Sci. U.S.A* 87, 2511-2515), incubated for 60-90 min with either heparinase III (25° C, pH 7.5; Sigma), chondroitinase ABC (37° C, pH 8.0; Sigma) or with *C. perfringens* neuraminidase (37° C, pH 6.0; Sigma). Cells were washed three times after treatment and resuspended in complete malaria medium, with 10% serum. Control erythrocyte suspensions were mock treated, washed and incubated as above.

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Results

Identification of a novel rosetting PfEMP1-variant

With the aim of identifying and isolating a PfEMP1-variant of a rosetting parasite, a single-cell RT-PCR assay was developed using micro-manipulation. Since the PfEMP1 messages are mostly expressed at ring stage, it is a difficult task to amplify cDNA from single trophozoites. However, the present inventor was successful in four out of eight pRBC studied and, when amplified, it was always a 434-bp sequence that was found (Fig. 1B). This was confirmed by amplification of *var* transcripts from bulk cultures, where the 434-bp amplicate was again detected unique to the rosetting *P. falciparum* and was not found present in non-rosetting parasites (Fig. 1C). Both the single cell- and the RT-PCR with the total RNA from the bulk cultures were repeated several times to make sure that the results were reproducible. Further, the 434-bp product was also found present in 7 out of 12 ring stage-parasites using single-cell RT-PCR (data not shown). Sequence analysis revealed that the amplified product encoded the semi-conserved sequence of the 5' located DBL-I domain of the *var* genes. Ten 434-bp sequences obtained from separate amplifications were found to be identical. 9 distinct PCR-amplified *var* transcripts from non-rosetting parasites were also isolated subcloned and sequenced. They were in all instances different from the 434-bp sequence (not shown). Northern-blot analysis with mRNA from the FCR3S1.2 clone and the 434-bp sequence as the labelled probe revealed a transcript of about ≈7.5-kb,

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the difference with the size of the coding cDNA of 6.7 kb being accounted for by a relatively large untranslated 5' region (Sundström et al, unpublished). The weak hybridisation seen with mRNA from R-parasites is probably due to hybridization to a different transcript (Fig. 1D). Thus, according to the present invention, a unique
5 PfEMP1 transcript has been found in rosetting parasites.

cDNA structure of rosetting PfEMP1

The entire coding region of the *var*- transcript containing the 434-bp motif was assembled with the 13 overlapping fragments and the coding sequence was found to be composed of 6684-bp (SEQ ID NO:1). 6684-bp encodes a 2228 aa polypeptide with an estimated molecular weight of 260 kDa. A single trypsin-sensitive polypeptide of a similar size was seen after ^{125}I - lactoperoxidase surface-labelling of the FCR3S1.2 (Fig.1E).

10 In an in-depth analysis of the expressed FCR3S1.2-*var1* transcript, it was found that the overall structure was similar to the published *var*-sequences. However, the sequence of the novel variant according to the present invention differs substantially from the prior sequences in that it is shorter than most previous sequences, as it contains two DBL-domains (DBL-1 and 4), rather than four, separated by a cysteine-rich interdomain region (CIDR) (Fig. 2B). Both the TM region and the negatively charged, acidic C-terminal segment (ATS) of the FCR3S1.2-*var1* exhibited a certain degree of homology (~80%) to previously published sequences. However, neither the potentially variable sequences of 434-bp *var*-gene fragment nor the other regions of the cDNA were contained in other published *var*-sequences, such as the one described in
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25 WO 96/33736.

Rosetting PfEMP1 contains clusters of glycosaminoglycan-binding motifs

The FCR3S1.2-*var1* sequence was examined for the presence of potential GAG-binding motifs. 19 potential GAG-binding sequences were identified and it was found that ~95% of them (18/19) were located in the N-terminus. 8/19 were situated in DBL-

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1, 5/19 in the CIDR C-terminally to DBL-1 and 5/19 in DBL-4. Only one potential
5 GAG-binding motif was seen beyond the TM region (SEQ ID NO:1). The N-terminal
distribution of these aa clusters is therefore consistent with the accessibility of the
FCR3S1.2-var1 at the surface of the pRBC. Thus, this may be the molecular
background to rosetting and thereby explain why rosettes are sensitive to heparin.

Rosetting PfEMP1 binds to a heparan sulfate-like GAG

To confirm the above findings experimentally, two domains of the FCR3S1.2-var1
transcript were subsequently expressed: one which had 8 GAG-binding motifs (DBL-
1, 1008-bp corresponding to 336 aa) and one which was the highly charged, acidic C-
terminal sequence (ATS) that lacks GAG-binding motifs (1353-bp corresponding to
451 aa). The purified DBL-1-GST efficiently bound to heparin-coupled sepharose
already after a few minutes at room temperature while the ATS-GST fusion protein did
not (Fig. 3B) and a second DBL-1-GST construct covering a distinct var sequence (var
2, Su, X.-Z, V.M. Heatwole, S.P. Wertheimer, F. Guinet, J.A. Herrfeldt, D.S. Peterson,
J.A. Ravetch, and T.E. Wellems. 1995. The large diverse gene family var encodes
proteins involved in cytoadherence and antigenic variation of *Plasmodium falciparum*-
infected erythrocytes. *Cell* 82, 89-100), which lacks GAG-binding motifs, also failed to
bind to the heparin-matrix (not shown). The adhesion could dose-dependently be
competed out with heparin or heparan sulfate but not with chondroitin sulfate, another
negatively charged erythrocyte surface expressed GAG (Fig. 3C). Taken together, this
indicates that the binding of DBL-1 to heparan sulfate is dependent on structure and
not merely ionic interactions between the two molecules. Heparan sulfate or a heparan
sulfate-like GAG therefore seems to be the binding target for the novel PfEMP1-
variant.

Rosetting PfEMP1 binds directly to erythrocytes

To study the potential binding of the recombinant PfEMP1 to erythrocytes we formed
monolayers of uninfected erythrocytes on glass-slides. The cells were subsequently
incubated with different concentrations of the fusion proteins. While the ATS-GST did

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not bind to the erythrocytes as detected with an anti-GST mAb, the DBL-1-GST gave a distinct surface staining of all the uninfected erythrocytes (Fig. 3D, E). This was titratable and could be inhibited with small amounts of heparin or heparan sulfate but not with the related GAG, chondroitin sulfate which suggests that the binding was specific. Heparan sulfate has previously been suggested to be present on human erythrocytes (Trybala, E., B. Svennerholm, T. Bergström, S. Olofsson, S. Jaensson, and J.L. Goodman. 1993. Herpes simplex virus type 1-induced hemagglutination: glycoprotein C mediates virus binding to erythrocyte surface heparan sulfate. *J. Virol.*, 67, 1278-1285, Baggio, B., G. Marzaro, G. Gambaro, F. Marchini, H.E. Williams, and A. Borsatti. 1990. Glycosaminoglycan content, oxalate self-exchange and protein phosphorylation in erythrocytes of patients with idiopathic' calcium oxalate nephrolithiasis. *Clinical Science*, 79, 113-116) and we therefore conclude that the DBL-1-GST fusion protein binds with heparan sulfate specificity to both to the solid-phase matrix as well as to normal erythrocytes.

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Recombinant DBL-1 disrupts rosettes and blocks rosette-reformation

The effect of the DBL-1 fusion protein on pre-formed rosettes was studied in order to confirm the biological role of PfEMP 1 in rosetting. Aliquots of a highly rosetting FCR3S1.2 culture was incubated with decreasing concentrations of DBL-1-GST or ATS-GST. DBL-1 caused a dose-dependent rosette reversion with 40-50% reversion at ≈50 µg/ml while the ATS-GST did not show any effect (Figure 4). The rosettes were not reformed upon prolonged incubation.

25

Rosetting is dependent on heparan sulfate

To establish the role of heparan sulfate also in rosetting we studied the disruptive activity of different GAGs on FCR3S1.2 rosettes. FCR3S1.2 rosettes were sensitive to both heparin and to heparan sulfate, but neither chondroitin sulfate A, keratan sulfate, nor hyaluronic acid had any effect on the rosettes (Fig. 5A). Chondroitin sulfate B had a slight effect only at high concentrations (Fig. 5A). These findings were confirmed by

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enzyme treatment of the uninfected erythrocyte: heparinase, but not chondroitinase or neuraminidase, treatment blocked the binding (Fig. 5B).

EXAMPLE 2

5 Materials and methods

Parasites and rosetting - Two distinct highly rosetting *P. Falciparum* laboratory strains were used: FCR3S1 (previously named R⁺PA1) and TM 284 (isolated in Thailand, 1990). Parasites were cultivated according to standard procedures (Udomsangpetch *et al.* 1989). Field isolates were obtained from patients attending the Albert Schweitzer Hospital, Lambaréné, Gabon between April and June 1996. Patients with a *P. falciparum* asexual stage parasite count superior to 10.000/ μ l blood were included in the study. A venous or capillary blood sample was taken after patients or parents informed consent. Blood was washed three times with RPMI and cultivated according to standard procedures (Udomsangpetch *et al.* 1989). Assessment for parasite growth and rosetting was made every 6-8 hours using a Leitz UV-light microscope. Briefly, an aliquot of parasite culture was mixed with a small amount of acridine orange and the sample was analyzed. Assays were performed on rosetting isolates when the majority of parasites in culture had reached the trophozoite stage. The rosetting rate was expressed as the number of infected erythrocytes in rosettes relative to the total number of late stage infected erythrocytes. Three hundred infected cells were counted. Parasitemia was calculated as the number of infected erythrocytes relative to the number of uninfected erythrocytes.

Saccharides - A number of GAGs, polysaccharides, heparin mono- and disaccharides were assessed for rosette disrupting activity. Heparin, heparan sulfate, chondroitin sulfate, dermatan sulfate (all from porcine intestine) as well as fucoidan (*Fucus vesiculosus*), dextran sulfate (Mol. Wt. 8.000), keratan sulfate (bovine cornea), hyaluronic acid (rooster comb), heparin/HS mono- and disaccharides (produced by digestion of heparin and HS by various heparinases) were purchased from Sigma (St. Louis, USA). Dextran (Mol. Wt. 60.000) was from Medisan Pharmaceuticals,

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Stockholm, Sweden. High molecular weight heparin with low affinity for antithrombin III (HMW-LA heparin) was produced by gel filtration of standard heparin on Sephadex S-200 (Pharmacia, Uppsala, Sweden) followed by passage over an antithrombin-Sepharose 4B column (Carlson *et al.* 1992). Modification of bovine lung heparin and structural characterizations were performed as described (Feyzi *et al.* 5 1997).

Treatment of erythrocytes and infected erythrocytes - Treatments of blood group O Rh⁺ red blood cells (RBC) and cultures were performed with heparinase III (0.033 IU/ml, 10 25° C, pH 7.5), chondroitinase ABC (20 IU/ml, 37° C, pH 7.5), *Clostridium perfringens* neuraminidase (0.1 IU/ml, 37° C, pH 6.0) and phospholipase C (0.1 IU/ml, 30° C, pH 7.5). The enzymes were purchased from Sigma and Seikagaku Corporation. Rosetting rate was assessed after 210 minutes incubation with enzyme, without changing any conditions in the culture and was compared to mock treated control cultures. No extracellular parasites or RBC gross morphology change was 15 observed at concentrations and incubation time used. Digestion with trypsin (100 IU/ml, 37° C, pH 7.5) and chymotrypsin (100 IU/ml, 37° C, pH 7.5) was stopped with soybean protease inhibitor (Sigma) after 20 minutes incubation and rosetting rate was assessed. Sodium periodate in RPMI was added to cultures and uninfected RBC at 10 mM concentration. Rosetting rate was checked after 15 minutes incubation at room 20 temperature and compared to mock treated control cultures.

Rosette disruption assays - Assays on wild isolated were performed on cultures 25 presenting a rosetting rate superior to 20% (range 23.6-86.2%, mean 48.6%) and parasitemia 1-10%. For laboratory strains, parasite cultures with rosetting rates of 50-70% and parasitemia 5-8% were used Aliquots (12.5-25 µl) of parasite cultures were mixed with equal amounts of sulfated glycoconjugate compound in RPMI in a 96-well microtiter plate, at final concentrations indicated. After incubation for 30 minutes at 37° C, assessment for rosetting was made as described above. The rosetting rate was 30 compared to that of culture mixed with RPMI.

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Competition assay using carboxy-fluorescein diacetate (C-FDA) labelled erythrocytes

- In order to measure the relative capacity of treated RBC to form rosettes, a competition assay was performed as described by Carlson and Wahlgren (Carlson *et al.* 1992), with some modifications. Blood group O Rh⁺ RBC were pre-treated for 60-90 min by enzymes or periodate as described above and mixed with a small amount of C-FDA (Sigma) stock solution to a final concentration of 250 µg/ml. The cells were incubated for 10-15 minutes at room temperature, then washed three times with RPMI and finally resuspended at 5% hematocrit in malaria culture medium containing 10% AB⁺ serum. Controls were mock treated and labelled as described above.

Fresh rosetting cultures grown in blood group O Rh⁺ RBC from the same donor were resuspended as above. A 25 µl aliquot of culture was mixed with an equal volume of the C-FDA-labelled uninfected RBC that had been subjected to enzyme or periodate pre-treatment. The rosettes were disrupted mechanically by drawing the cells through an injection needle (diameter 0.60 mm) 6-8 times. After 30 minutes incubation rosette reformation was assessed and the proportion of labelled to unlabelled RBC in rosettes calculated. Assays were considered valid when the ratio of labelled/unlabelled RBC in the assay was 0.9-1.1 and the ratio of labelled/unlabelled RBC participating in rosettes was 0.9-1.1 for control samples.

RESULTS

Disruption of rosettes from laboratory strains FCR3S1 and TM284 by SGs - Rosette disruption by GAGs and three other related polysaccharides (dextran, dextran sulfate and fucoidan) performed on two laboratory strains revealed differences in GAG-sensitivity (Table I). Although having a similar molecular structure, some compounds totally lacked rosette disrupting activity at high concentrations (1-10 mg/ml), while other compounds were potent rosette disrupters even at lower concentrations (1-10 µg/ml; Table I and not shown). FCR3S1 exhibited high sensitivity for heparin and HS, whereas dermatan/chondroitin sulfate had little or no effect. On the other hand, TM

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284 exhibited relatively higher sensitivity for dermatan/chondroitin sulfate than FCR3S1 and was less sensitive to HS/heparin (Table I). Still, heparin disrupts 80% of TM 284 rosettes at 1 mg/ml (not shown).

5 *Sensitivity of wild isolates to SGs* - Thirtytwo patient isolates were characterized in order to analyze the properties of fresh *P. Faciparum* parasites. The sensitivity of rosettes towards the different GAGs and other sulfated polysaccharides was clearly isolate dependent. In general, two major groups could be discerned (Fig. 1). One group was more sensitive to the glucosaminoglycans heparin and heparan sulfate, and somewhat less to the galactosaminoglycans (chondroitin sulfate and dermatan sulfate). For the second group of isolates the galactosaminoglycans were more effective competitors than the glucosaminoglycans. For both groups, fucoidan was a strong rosette disrupter in contrast to dextran sulfate which was much less effective than GAGs in most of the strains. This picture indicated that charge was an important but not the only determinant in rosette disruption. On the whole collection of wild isolates , fucoidan was the most effective rosette disrupter for most isolates and chondroitin sulfate the least effective, whereas dermatan sulfate, heparin, HS and dextran sulfate had an intermediate position. This was also true at other concentrations tested (10-100 µg/ml; not shown). No clear correlation was observed between differences in SG sensitivity of the isolates and parasitemia, rosetting rate or ABO blood group of the patient (not shown).

20 *Disruption of rosettes by modified heparin* - In order to investigate the relative importance of different molecular features, modified heparin was tested for rosette disruption on the heparin/HS sensitive FCR3S1 strain. When *N*-sulfate groups were replaced by the charge neutral acetyl group, heparin was ~ 100 fold less efficient in rosetting disruption compared to the native form (Fig. 2). In contrast to this marked effect, *O*-desulfation seemed to be much less important, as removal of either 2-*O*- or 6-*O*-sulfate groups resulted in an ~ 10 fold reduction of disruption efficiency. Even the

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most completely *O*-desulfated sample with only about 30% of disaccharide units still 2-*O*-sulfated had a similar order of efficiency.

Sensitivity of rosettes to disaccharides - As the effect of heparin seemed to be strongly related to the presence of the *N*-sulfate group, we tested whether the individual disaccharides would have a rosette reverting capacity. Different disaccharides that are gained by heparinase cleavage of heparin and HS were tested in the rosetting assay. The most active ones all contained the *N*-sulfated glucosamine, while the efficiency of the disaccharides was reduced by acetylation of the amino group corroborating data with the modified heparins (Fig. 3). The *O*-sulfation on the other hand had only limited influence. While 6-*O*-sulfation seemed to be less important, the 2-*O*-sulfate had some positive effect in addition to the *N*-sulfate. Additionally, heparin disaccharides II-H (Δ HexA-GlcN(6-OSO₃)), III-H (Δ HexA(2-OSO₃)-GlcN), and IV-H (Δ HexA-GlcN), all lacking *N*-sulfation and with a positively charged amino group, exhibited no rosette disrupting effect (not shown).

One active and one inactive disaccharide were studied in detail: complete rosette reversion was obtained by the *N*-sulfated disaccharide IV-S (Δ HexA-GlcNSO₃ at 45 mM while the *N*-acetylated disaccharide IV-A (Δ HexA-GlcNAc) completely lacked effect (Fig. 4A).

In a preliminary experiment the most predominant heparin disaccharide recovered after heparinase digestion, I-S (Δ HexA(2-OSO₃)-GlcNSO₃(6-OSO₃)), was titrated against heparin for rosette disruption on strain FCR3S1. At a concentration of 30 mM disaccharide, the rosettes were disrupted to the same extent as with heparin at 1 mg/ml (corresponds to \sim 70 μ M heparin molecules), e.e. the heparin chain was around 20 fold more efficient on a weight basis and around 500 fold more efficient on a molar basis. When wild type isolates were tested with this concentration of disaccharide, in comparison with the other competitors, it exhibited a similar pattern as heparin (Fig. 1).

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Sensitivity of rosettes to monosaccharides - When glucosamine was tested, the *N*-sulfated monosaccharides gave ~50% rosette reversion at 50 mM and additional sulfate groups and increasing negative charge did not potentiate rosette disruption. Some activity could also be attributed to *N*-acetylated glucosamine with an *O*-sulfate ester at C-6 (Fig. 5B). Similar results were obtained when the TM 284 strain was tested, though slightly higher concentrations were required (not shown). Also, *N*-sulfated galactosamine (not present in any GAG) lacked rosette disrupting activity, whereas *N*-sulfated glucosamine (common in heparin/ HS) caused total rosette disruption (Fig. 4B).

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Glycan oxidation and enzymatic treatment of whole cultures - Different treatments of infected erythrocyte cultures caused changes in rosetting behaviour without any gross change on RBC morphology on both strains tested (Fig. 6A). When the cultures were treated with the proteases trypsin or chymotrypsin rosetting was abolished completely, indicating that either receptor or ligand in the adhesion complex is a highly protease-sensitive protein. Heparinase III, which selectively cleaves $\text{-4GlcNR}\alpha\text{-[1}\rightarrow\text{4]-GlcA1-}$ (where R can be either N-acetamido or N-sulfo) linkages of heparan sulfate chains (Linhardt *et al.* 1990) resulted in a drastic reduction of the rosetting capacity of FCR3S1 and a somewhat lower decrease of TM 284 rosetting. Chondroitinase ABC, cleaving $\text{GalNAc}\alpha\text{-[1}\rightarrow\text{4]-GlcA}$ bonds in chondroitin sulfate had only effect on the rosetting of the TM284 strain. No effect was seen by either neuraminidase or phospholipase C treatment. Sodium periodate which would oxidize any glycan type with vicinal hydroxyl groups had an intermediate effect on both parasite strains.

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Glycan oxidation and enzymatic treatment of uninfected RBC - In order to assess whether above results can be attributed to the infected RBC *per se* or to the uninfected RBC that participate in rosetting, competition assays were achieved with pre-treated, C-FDA labelled uninfected RBC. Sodium periodate treatment of uninfected RBC decreased their rosette binding capacity 4 times compared to non-treated RBC. The rosette binding capacity of heparinase III treated RBC was also decreased 4-5 fold.

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Treatment with heparinase III in presence of soybean protease inhibitor did not reverse the effect, and addition of HS to heparinase completely blocked the heparinase activity (not shown). Also, trypsin and chymotrypsin treatment affected the binding capacity, with a 2-5 fold decrease, but less than the treatment of the whole culture. This finding
5 suggests that the primary protease sensitive molecule is the ligand on the infected RBC, while not all receptor structures on the uninfected RBC are sensitive to proteolytic degradation. This can be either due to a reduced protease sensitivity of glycosylated proteins/proteoglycans or because the receptor glycans would sit on a non-proteinaceous core. For the TM 284 strain, a two fold decrease of binding capacity was observed after chondroitinase ABC treatment, whereas FCR3S1 was
10 unaffected (Fig. 6 B).

DISCUSSION

The present invention discloses the identification of a novel PfEMP1-variant as the rosetting ligand and heparan sulfate, or a heparan sulfate like molecule, as the rosetting receptor. A single cell RT-PCR technique was developed in order to investigate the association of expression of the novel PfEMP1-variant with rosetting-binding at the single cell level. The PfEMP 1 messages were found to be present in ring stage parasites, in trophozoites and in schizonts, although it was less abundant in the more mature stage parasites. However, these were studied in great detail as they do form rosettes and rings do not. One PfEMP 1 mRNA species was amplified from single rosetting parasites and the appearance of the same fragment in amplifications with total RNA from R⁺ bulk cultures, but not with total RNA from non- or low rosetting parasites, indicated that the message was unique to rosetting parasites. It was evident,
15 however, that the parasites were not homogeneous even in cultures of a high rosetting rate suggesting that the non-rosetting parasites may express different PfEMP 1s. Similarly, although a strong hybridisation to a ≈7.5 kb message from the rosetting parasites was found, there was still a small hybridisation to a slightly smaller species with RNA from the R⁻ parasites. This probably reflects the transcription of a different
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var gene, since the FCR1S.2 *var1* sequence was not found in the amplified products from R- parasites.

Cloning of full length cDNA from eukaryotic cells is always difficult. For example, 5 Harshman *et al.* (1995) *Hum. Mol. Genet.* 4: 1259-1266, have shown that different methods of identifying cDNAs from a genomic region result in a surprisingly different array of candidate genes. To be able to achieve this, the RT-PCR parameters were first optimised, so that most PfEMP1 transcripts could be amplified. With these conditions and a primer sequence from the unique 434-bp rosetting PfEMP1 cDNA, one large 10 single down-stream fragment of 4.9-kb of cDNA was amplified, which upon the digestion with EcoR I and Hind III was fragmented into distinct bands indicating that the amplified product was composed of one sequence. The 4.9-kb cDNA species was subsequently confirmed to be a single amplificate by sequencing. Rosetting-PfEMP1 specific sequences of both the variable region, upstream to the trans-membrane 15 domain, and of the 434-bp fragment were used to obtain the sequences of overlapping regions of the 3' and the 5' regions of this transcript. Five overlapping fragments were sequenced to ascertain the 3' overlap and seven to ascertain the 5' overlap. The correctness was further checked by RT-PCR with specific primers flanking the overlapping regions after the assembly of the entire coding sequence. Each of the 14 20 PCR-amplified fragments were sequenced in both directions and the overlapping fragments were found to be correct and identical in all regions. It is therefore, hypothesized that the assembled transcript is indeed derived from one single gene.

The high sensitivity of rosettes to heparin and above specified glycosamino glycans 25 (GAGs) made the present inventors speculate that the parasite uses sulfated carbohydrates as the rosetting receptor. The appearance of potential GAG binding motifs in this novel PfEMP 1 sequence was therefore checked after the assembly of the transcript. Clusters of GAG binding motifs, the consensus sequence of which have previously been proposed by others, were then identified. All the motifs showed an 30 identical or a similar composition as other published heparin or heparan sulfate binding

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motifs found in both malaria parasites and other organisms (Sinnis, P., P. Clavijo, D. Fenyö, B.T. Chait, C. Cerami, and V. Nussenzweig. 1994. Structural and functional properties of region II-plus of the malaria circumsporozoite protein. *J. Exp. Med.* 180, 297-306, Jackson, L.R., J.S. Busch, and D.A. Cardin. 1991. Glycosaminoglycans: molecular properties, protein interactions, and role in physiological processes. *Physiol. Rev.*, 71, 481-530). The expressed DBL-1, which had 8 motifs, bound to heparin-sepharose, to the membrane of normal RBC, disrupted naturally formed rosettes and blocked rosette-reformation while the ATS fusion protein did not. The inhibitory activity of heparan sulfate as well as the inability of chondroitin sulfate to inhibit or block the attachment informed us that it was the molecular structure of the GAG that is important for binding. In complementary experiments, the DBL-1 region of a second var-gene transcript (*var-2*) which lacks GAG-binding motifs was expressed and found not to bind to the heparin-sepharose (Sahlen, not shown), which also support a functional role of the GAG binding sequences in the rosetting DBL-1.

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The rosette reversion obtained by the addition of recombinant DBL-1 to naturally formed rosettes indicated that the parasite uses the novel PfEMP1-variant as the main rosetting ligand. The deletion of rosetting by heparinase treatment of normal RBC provided further confirmation about this specific ligand-receptor interaction, indeed suggesting that heparan sulfate, or at least a heparan sulfate-like molecule, is involved in the binding. Heparinase treatment also disrupted the rosettes of other strains of parasites (TM180, TM284), while the rosettes of the strain R29 were not affected (not shown), findings which are in concordance with those of Rowe *et al* (Rowe, J.A., J.M. Moulds, C.I. Newbold, and L.H. Miller. 1997. *P. falciparum* rosetting mediated by a parasite-variant erythrocyte membrane protein and complement-receptor 1. *Nature* 388:292-295). In separate experiments, heparin-derived mono- and di-saccharides which were of the same net charge but had their sulfate groups at different positions were studied for their rosette-disrupting activities. Those with the sulfate groups in the same position as heparan sulfate inhibited binding while the others did not (Barragan unpublished and see above). Further, while the N-sulfated mono-saccharide

glucosamine, a component of heparan sulfate, had a good anti-rosetting activity the identically N-sulfated galactosamine had none (Barragan et al, unpublished). Heparin has also been found to disrupt rosettes from 27 out of 54 fresh *P. falciparum*-isolates (50%) suggesting that heparan sulfate is used by these parasites as rosetting receptors

5 (Carlson, J., H.P. Ekre, H. Helmby, J. Gysin, B.M. Greenwood, and M. Wahlgren.

1992. Disruption of *Plasmodium falciparum* erythrocyte rosettes by standard heparin and heparin devoid of anticoagulant activity. *Am. J. Trop. Med. Hyg.* 46, 595-602). The receptors used by the heparin-resistant fresh isolates maybe other GAGs, as it has been found that rosettes of most parasites may be disrupted by either one or the other of the human GAGs (Barragan, unpublished). Further, other sulphated glycans, such as fucoidan, disrupted rosettes of almost all fresh isolates (Fig. 1). Thus, according to the present invention, it has been found that FCR3S1.2 uses heparan sulfate, or a heparan sulfate like molecule, on the erythrocyte surface as a rosetting receptor and that the other GAGs may be receptors of other parasites.

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Heparan sulfate is a molecule presents on all cells, including erythrocytes (Trybala, E., B. Svennerholm, T. Bergström, S. Olofsson, S. Jaensson, and J.L. Goodman. 1993.

Herpes simplex virus type 1-induced hemagglutination: glycoprotein C mediates virus binding to erythrocyte surface heparan sulfate. *J. Virol.*, 67, 1278-1285, Baggio, B., G.

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Marzaro, G. Gambaro, F. Marchini, H.E. Williams, and A. Borsatti. 1990.

Glycosaminoglycan content, oxalate self-exchange and protein phosphorylation in erythrocytes of patients with idiopathic' calcium oxalate nephrolithiasis. *Clinical Science*, 79, 113-116); 4×10^6 molecules have been suggested to be expressed at the surface of normal hepatocytes (Kjellen, L., Å. Oldberg, and M. Höök. 1980. *Cell*

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surface heparan sulfate. *J Biol. Chem.* 21, 10407-10413). The density on the RBC should also be high if this indeed is the receptor used by the parasite for rosetting. The involvement of GAGs as receptor structures for other cell-to-cell interactions of *P. falciparum* has previously been suggested e.g. for endothelial binding , for liver- and erythrocyte invasion (Sinnis, P., P. Clavijo, D. Fenyö, B.T. Chait, C. Cerami, and V.

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Nussenzweig. 1994. Structural and functional properties of region II-plus of the

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malaria circumsporozoite protein. *J. Exp. Med.* 180, 297-306, Kulane, A., H-P. Ekre, P. Perlmann, L. Rombo, M. Wahlgren, and B. Wahlin. 1992. Effect of different fractions of heparin on *Plasmodium falciparum* merozoite invasion of red blood cells *in vitro*. *Am. J. Trop. Med. Hyg.*, 46, 589-594, Robert, C., B. Pouvelle, P. Meyer, K.

5 Muanza, A. Scerf, and J. Gysin. 1995. Chondroitin-4-sulfate (proteoglycan) as *Plasmodium falciparum*-infected erythrocyte adherence receptor of brain microvascular endothelial cells. *Res. Immunol.* 146, 383-393, Rogerson, S.J., S.C. Chaiyaroj, K. Ng, J.C. Reeder, and G.V. Brown. 1995. Chondroitin sulfate A is a cell surface receptor for *Plasmodium falciparum*-infected erythrocytes. *J. Exp. Med.* 182, 15-20). Taken together this may indicate that the parasite has adapted a more general strategy for interacting with the host by using the negatively charged proteoglycans as receptors exposed on the exterior of every cell surface.

10 15 According to the present invention, it has also been found that the rosette disruption capacity is not only determined by the molecular weight of SGs. Hyaluronic acid, by far the largest of all tested molecules, exhibited no rosette disrupting activity while smaller molecules did. Also, an association between average negative charge and rosette reverting capacity was not always observed, e.g. hyaluronic acid which has a higher average negative charge density than fucoidan did not exhibit a rosette-disrupting activity while fucoidan did. Comparison of heparin and fucoidan, on the other hand, suggests that the effect of correct positioning within a three dimensional structure could be much more essential than the average amount of negative charge which is clearly lower in fucoidan (Patankar *et al.* 1993). An association to negative charge could be the explanation for differences between heparin and HS, although 20 more specific sequence determinants can not be excluded.

25

When assaying the heparin/HS-sensitive FCR3S1 strain it is apparent that modified heparin preparations with reduced overall degree of sulfation, and thus negative charge, loose competition capacity though not in a strictly charge dependent manner. 30 This finding supports the conclusion that other determinants must be of importance for

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the binding to infected erythrocytes. *N*-desulfated heparin preparations were at least one hundred times weaker rosette reverters compared to heparin. In contrast to the effect of *N*-desulfation, *O*-desulfation seemed to be connected with minor reduction of competition efficiency, indicating the importance of the type of sulfate, *i.e.* *N*-sulfation.

5 An analogous conclusion may be drawn by comparison of the more efficient rosetting reverter heparin as compared to HS. One of the basic differences between heparin and HS is the degree and distribution of the *N*-sulfate groups, which in turn leads to a predominant localization of *O*-sulfation to *N*-sulfated blocks (Salmivirta *et al.* 1996). *N*-sulfation reaches 40 - 60 % of all the disaccharides in an average HS chain (Maccarana *et al.* 1996) while the value for heparin is over 90 %. Indeed, the difference in competition effect of heparin and HS in rosetting could be explained by the more abundant presence of *N*-sulfation in heparin.

10 15 To further test the importance of sulfation position over anionic feature of a carbohydrate, heparin/HS derived mono- and disaccharides were assayed for rosette disrupting activity. Twenty-nine wild isolates were tested for sensitivity to heparin and heparin disaccharide I-S (Δ HexA(2-OSO₃)-GlcNSO₃-(6-OSO₃)), and the sensitivity pattern obtained was very similar for the two compounds at equipotent concentrations, whereas it could differ for other GAGs and polysaccharides. Our results talk therefore 20 in favour of an identical site of action for heparin and the heparin disaccharide I-S suggesting that results with mono- and disaccharides reflect at least part of the heparin/HS binding site(s) on infected erythrocytes.

25 Molecular net negative charge can not by itself explain the anti-rosetting activity: some highly negatively charged monosaccharides exhibited no rosette disrupting effect, while some monosaccharides with one single negative charge disrupted rosettes. Also, the fact that higher degree of sulfation did not increase rosette reverting potency, corroborates that it is the position of the sulfate group(s) rather than the number that is the determinant. In case of the disaccharides, 6-*O*-sulfation seems to be less important, 30 while the 2-*O*-sulfate has some positive effect in addition to the *N*-sulfate group. This

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could be explained by rotational freedom of the glycosidic linkage which results in the possible exposure of both sulfate groups on the same side of the sugar plane, while the 6-O-sulfate would protrude on the opposite side. Also, *N*-sulfated glucosamine (present in heparin and HS) abolishes rosetting, whereas its stereoisomer *N*-sulfated galactosamine (not present in any GAG) does not, all emphasizing that the *N*-sulfated glucosamine is part of the binding sequence.

To analyze whether competition by heparin and heparin derived oligosaccharides reflects the existence of a heparin/HS-like ligand on the erythrocytes, whole cultures and uninfected erythrocytes were treated by different glycan degrading procedures. For the heparin/HS sensitive strain FCR3S1 a clear reduction of rosette formation was provoked by either unspecific glycan oxidation through periodate or a selective digestion of heparin/HS chains by heparinase (Fig. 6). Rosetting was not hampered by either chondroitinase, neuraminidase or phospholipase C treatment of cultures, supporting the view that the ligand is of heparin/HS nature. Proteolytic treatment of the cultures, on the other hand, indicated that protein components are involved in the rosetting mechanism as well, either as lectin-like ligands (i.e. on the infected erythrocyte) or as carrier of the glycan epitopes (i.e. on the uninfected erythrocyte). Rosetting by strain TM 284 seemed to be more sensitive to competition by chondroitin sulfate and dermatan sulfate, although higher amounts of heparin/HS were also effective. This observation was confirmed by enzymatic treatment of the erythrocytes as chondroitinase had a profound effect on the rosetting.

The separate treatment of uninfected erythrocytes was performed in order to establish whether the infected erythrocyte or the rosetting uninfected erythrocytes contain specific features involved in rosetting. The higher sensitivity of the uninfected erythrocytes for glycan destruction would indicate that glycan structures constitute parts of the rosetting receptor(s). The opposite argumentation would hold true for the proteolytically destroyed component which seems to sit on the side of the infected erythrocytes, although also a part of the glycans could be removed by proteolytic

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cleavage of their core proteins. However, the infected erythrocytes seem to be much more sensitive for the proteolytic degradation as compared to glycan oxidation.

We have previously suggested that rosetting may be mediated by lectin-like interactions, with non-obligate involvement of different ABO-blood group antigens (Carlson *et al.* 1992). CD36 has also been attributed a receptor function (Handunnetti *et al.* 1992) and recently, complementreceptor 1 was reported to play a role in the formation of rosettes (Rowe *et al.* 1997). The fact that heparin sensitivity decreases when the same parasites are cultivated in erythrocytes from their preferred ABO-blood group (Carlson *et al.* 1992) could indicate simultaneous involvement of more than one rosetting receptor and that heparin is not interacting with these receptors. A role for serum proteins in rosetting has also been shown (Scholander *et al.* 1996). Although an interaction of GAGs with serum factors can not be excluded, this effect should be of minor importance since strains with different serum dependency were equally affected (not shown). Furthermore, a recombinant fusion-protein covering the C-terminally located Duffy-like binding domain (DBL-1) of PfEMP1, adhered directly to erythrocytes and bound to a heparin-matrix, in the absence of serum factors (Chen *et al.* 1998).

As a conclusion, the present invention provides the identification, by single-cell RT-PCR and cDNA cloning, of a new adhesive ligand *Plasmodium falciparum* erythrocyte membrane protein-1-variant, PfEMP1. Rosettes of the novel PfEMP1-variant contains clusters of glycosaminoglycan-binding motifs. A recombinant fusion protein (DBL-1-GST) has been found to adhere directly to normal erythrocytes, disrupt naturally formed rosettes, block rosette-reformation and bind to a heparin-sepharose matrix. The adhesive interactions could be inhibited with heparan sulfate or enzymes that remove heparan sulfate from the cell surface while other enzymes or similar glycosaminoglycans of an alike negative charge did not affect the binding. Further, fucoidan, a sulfated glycan, has been shown to be particularly effective in disrupting rosettes. The novel PfEMP1-variant is suggested to be the rosetting ligand and

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carbohydrates, heparan sulfate, or a heparan sulfate-like molecule, the receptor both for PfEMP1 binding and naturally formed erythrocyte rosettes.

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CLAIMS

1. A carbohydrate which exhibits at least one negatively charged glycosamin-oglycan-like moiety, whereby it is capable of essentially specific binding to a malaria erythrocyte membrane protein or a functional analogue thereof.

5

2. A carbohydrate according to claim 1, wherein said at least one glycosamin-oglycan-like moiety is sulfated.

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3. A carbohydrate according to claim 1, wherein said at least one glycosamin-oglycan like-moiety is a heparan sulfate like moiety.

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4. A carbohydrate according to any one of claims 1-3, which more specifically is capable of essentially specific binding to at least one of the binding segments of the amino acid sequence disclosed in SEQ ID NO:1 or to a functional analogue thereof.

5. A carbohydrate according to any one of claims 1-4, which is capable of essentially specific binding to an amino terminal part of the amino acid sequence disclosed in SEQ ID NO:1 or to a functional analogue thereof.

20

6. A carbohydrate according to any one of claims 1-5, which is capable of essentially specific binding to essentially all of the binding segments of the amino acid sequence disclosed in SEQ ID NO:1 or to a functional analogue thereof.

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7. A carbohydrate according to any one of claims 1-6, which is capable of essentially specific binding to the sequence disclosed in SEQ ID NO:1 or to a functional analogue thereof.

8. A carbohydrate according to any one of claims 1-7 for use as a medicament.

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9. Use of a carbohydrate according to any one of claims 1-7 in the manufacture of a medicament against malaria.

10. A pharmaceutical composition comprising a carbohydrate according to any one of claims 1-7 in a pharmaceutically acceptable carrier.

11. A method of treating a patient suffering from a malaria infection comprising administering to the patient of an effective amount of the pharmaceutical composition according to claim 10.

12. A method according to claim 11, wherein the malaria infection is a *P. falciparum* infection.

13. An isolated polypeptide originating from a malaria erythrocyte membrane protein comprising an amino-terminal part of the sequence according to SEQ ID NO:1 or an analogue thereof.

14. A polypeptide originating from a malaria erythrocyte membrane protein comprising at least about 300 amino acids of the sequence according to SEQ ID NO:1 or a functional analogue thereof.

15. A polypeptide according to claim 13 or 14 comprising about 400-500 amino acids, preferably about 423 amino acids, of the sequence according to SEQ ID NO:1 or a functional analogue thereof.

16. A polypeptide according to any one of claims 13-15 capable of essentially specific binding to a negatively charged glycosaminoglycan-like moiety, preferably a sulfated glycosaminoglycan-like moiety.

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17. A polypeptide according to any one of claims 13-16 having a weight of about 100-300 kDa, preferably about 280 kDa.

18. A method of preparing a polypeptide according to any one of claims 13-17 or a functional analogue thereof, which comprises the steps of

- 5 (1) the inserting into an expression vector of a nucleic acid encoding said polypeptide or analogue thereof;
- (2) the transfection of a host cell capable of expressing said nucleic acid with said expression vector to express said polypeptide; and
- 10 (3) the recovery of the expressed polypeptide.

19. A nucleic acid encoding a polypeptide according to any one of claims 13-17.

20. A nucleic acid capable of specific hybridisation under stringent conditions to a nucleic acid according to claim 19.

21. A recombinant fusion protein comprising a polypeptide according to any one of claims 13-17.

20 22. A polypeptide according to any one of claims 13-17 for use as a medicament.

23. Use of a polypeptide according to any one of claims 13-17 in the manufacture of a medicament for the treatment or prevention of malaria, or the vaccination against malaria.

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24. A pharmaceutical composition comprising a polypeptide according to any one of claims 13-17 in a pharmaceutically acceptable carrier.

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25. A method of treating a patient suffering from a malaria infection comprising administering to said patient of an effective amount of the pharmaceutical composition according to claim 23.

5 26. A method according to claim 24, wherein the malaria infection is a *P. falciparum* infection.

10 27. Use of a polypeptide according to any one of claims 16-18 as a model substance for identifying substances binding to malaria erythrocyte membrane protein or analogues thereof.

28. An antibody which is specifically immunoreactive with a polypeptide according to any one of claims 13-17 or with an analogue thereof.

15 29. A pharmaceutical composition comprising an antibody according to claim 28 in a pharmaceutically acceptable carrier.

20 30. A method of treating a patient suffering from a malaria infection comprising administering to said patient of an effective amount of the pharmaceutical composition according to claim 29.

31. A method of preventing malaria in a human or animal object comprising exposure of said human or animal for an effective amount of the pharmaceutical composition according to claim 29.

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32. A method according to claim 30 or 31, wherein the malaria is *P. falciparum* malaria.

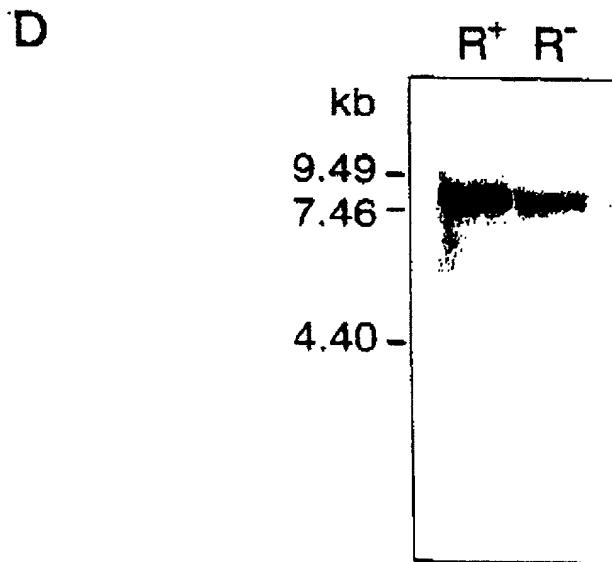
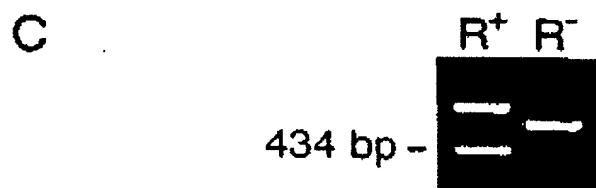
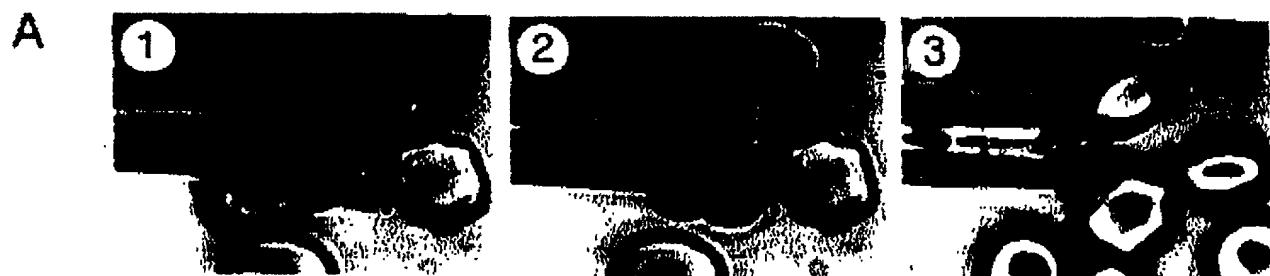
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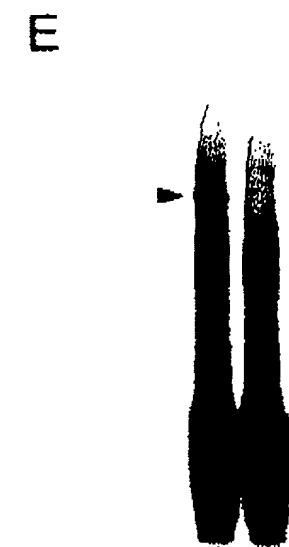
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Figure 1



Rosetting rate (%) 84 9

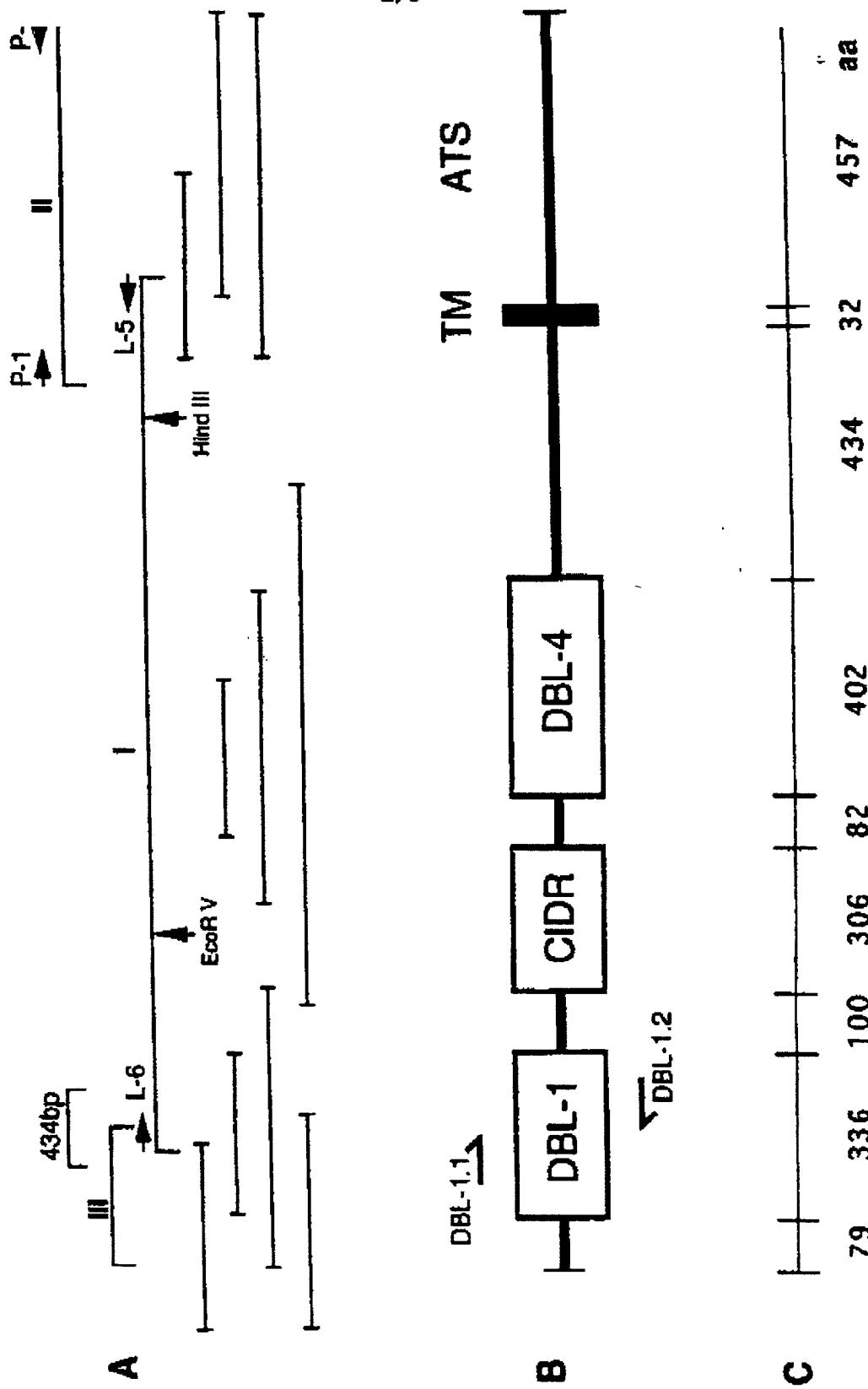


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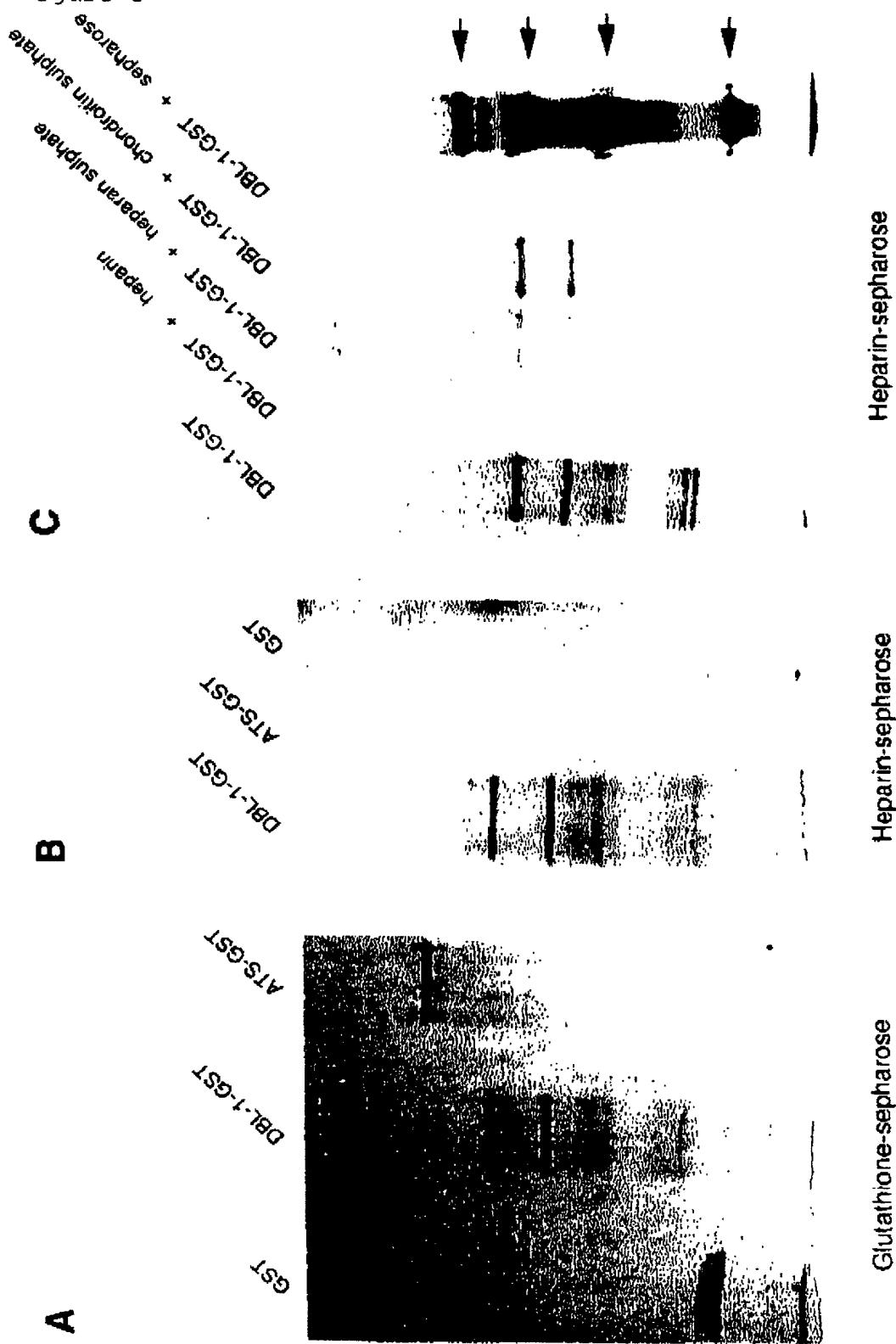
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Figure 3



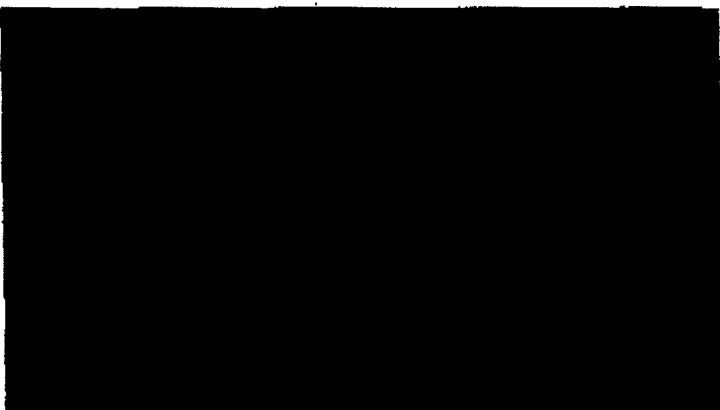
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Figure 3 (continued)

D**E**

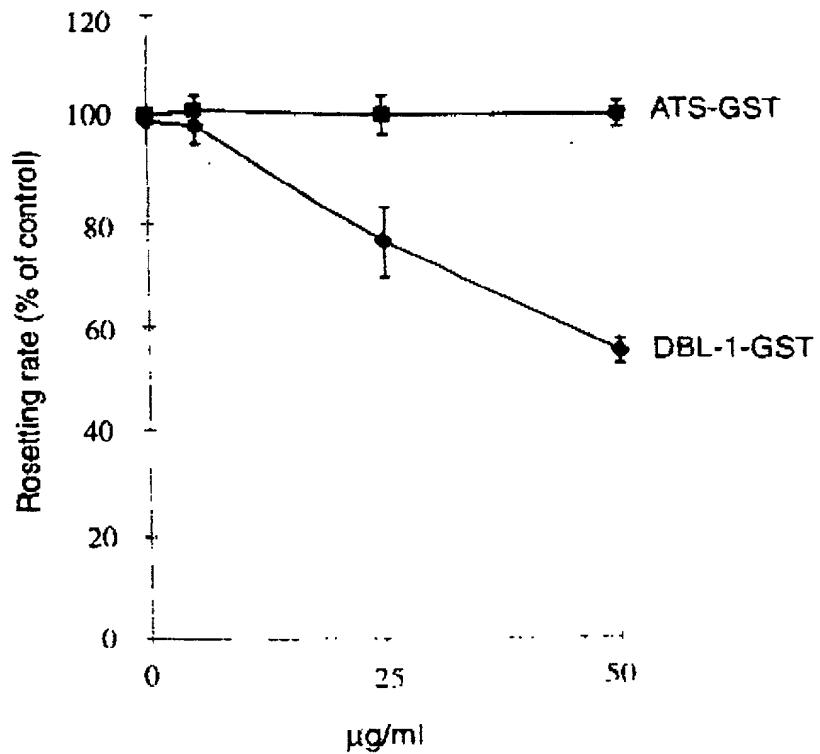
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Figure 4



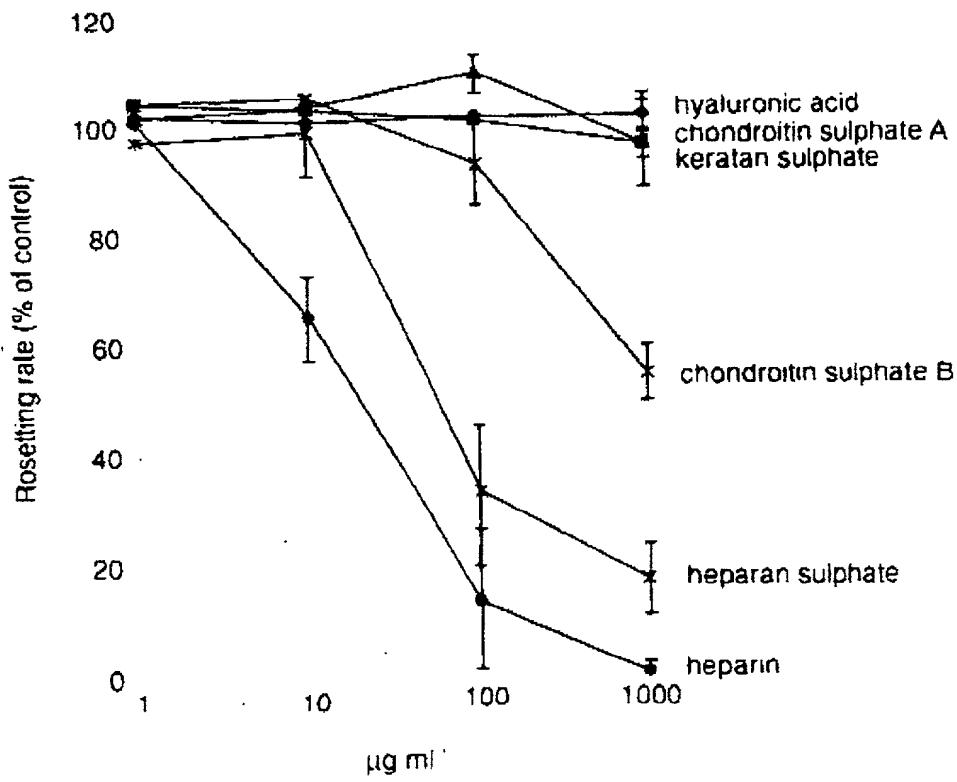
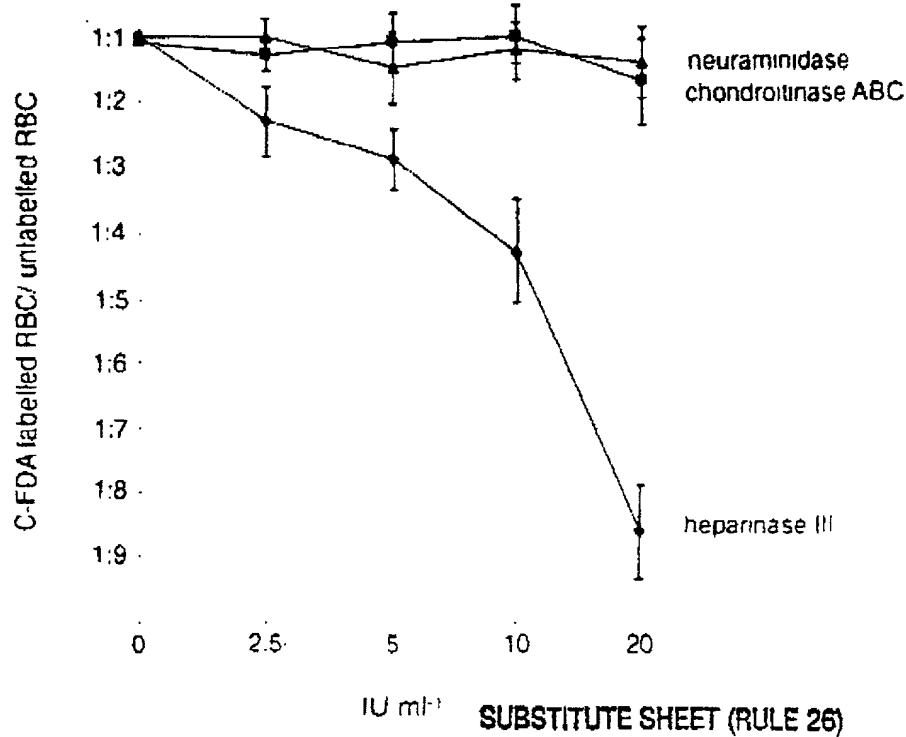
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Figure 5

A**B**

SUBSTITUTE SHEET (RULE 26)

45300-59676

COMBINED DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

MALARIA POLYPEPTIDES

the specification of which: *(check one)*

REGULAR OR DESIGN APPLICATION

is attached hereto.

was filed on _____ as application Serial No.
and was amended on (if applicable).

PCT FILED APPLICATION ENTERING NATIONAL STAGE

was described and claimed in International application No. PCT/SE98/01675 filed on 18 September 1998 and as amended on (if any).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

PRIORITY CLAIM

I hereby claim foreign priority benefits under 35 USC 119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed.

PRIOR FOREIGN APPLICATION(S)

Country	Application Number	Date of Filing (day, month, year)	Priority Claimed
Sweden	9703386-4	19 September 1997	yes

(Complete this part only if this is a continuing application.)

I hereby claim the benefit under 35 USC 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of 35 USC 112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37 Code of Federal Regulations §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

(Application Serial No.)

(Filing Date)

(Status—patented, pending, abandoned)

POWER OF ATTORNEY

The undersigned hereby authorizes the U.S. attorney or agent named herein to accept and follow instructions from Albihns Patentbyra Stockholm AB as to any action to be taken in the Patent and Trademark Office regarding this application without direct communication between the U.S. attorney or agent and the undersigned. In the event of a change in the persons from whom instructions may be taken, the U.S. attorney or agent named herein will be so notified by the undersigned.

As a named inventor, I hereby appoint the following attorney(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith: Robert J. PATCH, Reg. No. 17,355, Andrew J. PATCH, Reg. No. 32,925, Robert F. HARGEST, Reg. No. 25,590, Benoît CASTEL, Reg. No. 35,041, Eric JENSEN, Reg. No. 37,855, Thomas W. PERKINS, Reg. No. 33,027, and Roland E. LONG, Jr., Reg. No. 41,949, c/o YOUNG & THOMPSON, Second Floor, 745 South 23rd Street, Arlington, Virginia 22202.

Address all telephone calls to Young & Thompson at 703/521-2297.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

1 - Full name of sole or first inventor: Mats WAHLGREN
(given name, family name)

Inventor's signature Mats Wahlgren Date 16 March, 2000

Residence: Stocksund, Sweden EX

Citizenship: Swedish

Post Office Address: Ljungvagen 7,
S-182 79 Stocksund, Sweden

2 - Full name of second joint inventor, if any: Antonio BARRAGAN
(given name, family name)

Inventor's signature A. Barragan Date 16 March, 2000

Residence: Huddinge, Sweden EX

Citizenship: Spanish

Post Office Address: Byggmästarvägen 53,
S-141 73 Huddinge, Sweden

3 - Full name of third joint inventor, if any: Johan CARLSON
(given name, family name)

Inventor's signature J. Carlson Date 16 March, 2000

Residence: Stockholm, Sweden EX

Citizenship: Swedish

Post Office Address: Microbiology and Tumor Biology Center (MTC)
Karolinska Institutet, P.O. Box 280
S-171 77 Stockholm, Sweden

4 - Full name of fourth joint inventor, if any: Chen QIJUN
(given name, family name)

Inventor's signature Chen QIJUN

Date 16 March, 2000

Residence: Stockholm, Sweden M

Citizenship: Chinese

Post Office Address: Microbiology and Tumor Biology Center (MTC)
Karolinska Institutet, P.O. Box 280
S-171 77 Stockholm, Sweden

5 - Full name of fifth joint Inventor, if any: Victor FERNANDEZ
(given name, family name)

Inventor's signature Victor Fernandez

Date 16 March, 2000

Residence: Stockholm, Sweden M

Citizenship: Venezuelan

Post Office Address: Microbiology and Tumor Biology Center (MTC)
Karolinska Institutet, P.O. Box 280
S-171 77 Stockholm, Sweden

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

- (A) NAME: KAROLINSKA INNOVATIONS AB
- (B) STREET: TOMTEBOGATAN
- (C) CITY: STOCKHOLM
- (E) COUNTRY: SWEDEN
- (F) POSTAL CODE (ZIP): 171 77
- (G) TELEPHONE: +46-(0)8-728 65 10
- (H) TELEFAX: +46-(0)8-303423

(ii) TITLE OF INVENTION: MALARIA POLYPEPTIDES

(iii) NUMBER OF SEQUENCES: 1

(iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2228 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: N-terminal

(ix) FEATURE:

- (A) NAME/KEY: Binding-site
- (B) LOCATION: 79..86

(ix) FEATURE:

- (A) NAME/KEY: Binding-site
- (B) LOCATION: 102..107

(ix) FEATURE:

- (A) NAME/KEY: Binding-site
- (B) LOCATION: 201..207

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(ix) FEATURE:

- (A) NAME/KEY: Binding-site
- (B) LOCATION:221..232

(ix) FEATURE:

- (A) NAME/KEY: Binding-site
- (B) LOCATION:341..350

(ix) FEATURE:

- (A) NAME/KEY: Binding-site
- (B) LOCATION:377..382

(ix) FEATURE:

- (A) NAME/KEY: Binding-site
- (B) LOCATION:411..418

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- (A) NAME/KEY: Binding-site
- (B) LOCATION:533..549

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- (B) LOCATION:569..576

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- (B) LOCATION:646..651

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- (B) LOCATION:688..693

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- (B) LOCATION:941..949

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- (A) NAME/KEY: Binding-site
- (B) LOCATION:1032..1039

(ix) FEATURE:

- (A) NAME/KEY: Binding-site
- (B) LOCATION:1152..1159

(ix) FEATURE:

- (A) NAME/KEY: Binding-site
- (B) LOCATION:1170..1175

(ix) FEATURE:

- (A) NAME/KEY: Binding-site
- (B) LOCATION:1232..1239

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(ix) FEATURE:

(A) NAME/KEY: Binding-site
 (B) LOCATION: 1771..1777

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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His Val Leu Asp Glu Phe Gly Gln Lys Val His Asp Glu Val His Gly
 20 25 30

Glu Ala Lys Asn Tyr Val Ser Glu Leu Lys Gly Ser Leu Ser Leu Ala
 35 40 45

Ser Ile Leu Gly Glu Thr Ala Phe Thr Val Lys Ser Met Gln Thr Glu
 50 55 60

Ser Lys Tyr Thr Glu Leu Ile Glu Ala Asn Ser Lys Arg Asn Pro Cys
 65 70 75 80

Lys Lys Asp Gly Lys Gly Asn Asp Val Asp Arg Phe Ser Val Lys Glu
 85 90 95

Gln Ala Gly Tyr Asp Asn Lys Lys Met Lys Cys Ser Asn Gly Met Thr
 100 105 110

Cys Ala Pro Phe Arg Arg Leu His Leu Cys Asn Lys Asn Phe Pro Asn
 115 120 125

Met Asn Ser Asn Asp Ser Ser Lys Ala Lys His Asp Leu Leu Ala Glu
 130 135 140

Val Cys Met Ala Ala Lys Tyr Glu Gly Glu Ser Ile Lys Thr His Tyr
 145 150 155 160

Pro Lys Tyr Asp Ser Lys Tyr Pro Gly Ser Asp Phe Pro Met Cys Thr
 165 170 175

Met Leu Ala Arg Ser Phe Ala Asp Ile Gly Asp Ile Ile Arg Gly Arg
 180 185 190

Asp Leu Tyr Leu Gly Asn Lys Lys Lys Gln Asn Gly Lys Glu Thr
 195 200 205

Glu Arg Glu Lys Leu Glu Gln Lys Leu Lys Glu Ile Phe Lys Lys Ile
 210 215 220

His Asp Asn Leu Lys Asp Lys Glu Ala Gln Lys Arg Tyr Asn Gly Asp
 225 230 235 240

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Glu Asp Pro Asn Phe Tyr Lys Leu Arg Glu Asp Trp Trp Thr Ala Asn
 245 250 255

Arg Glu Thr Val Trp Gly Ala Met Thr Cys Ser Lys Glu Leu Asp Asn
 260 265 270

Ser Ser Tyr Phe Arg Ala Thr Cys Asn Asp Thr Gly Gln Gly Pro Ser
 275 280 285

Gln Thr His Asn Lys Cys Arg Cys Asp Lys Asp Lys Gly Ala Asn Ala
 290 295 300

Gly Lys Pro Lys Ala Gly Asp Gly Asp Val Thr Ile Val Pro Thr Tyr
 305 310 315 320

Phe Asp Tyr Val Pro Gln Tyr Leu Arg Trp Phe Glu Glu Trp Ala Glu
 325 330 335

Asp Phe Cys Arg Lys Lys Lys Leu Glu Asn Leu Glu Lys Gln
 340 345 350

Cys Arg Gly Lys Asp Lys Ser Asp Glu Tyr Arg Tyr Cys Ser Arg Asn
 355 360 365

Gly Tyr Asp Cys Glu Gln Thr Ile Ser Arg Lys Gly Lys Val Arg Met
 370 375 380

Gly Lys Gly Cys Thr Asp Cys Phe Phe Ala Cys Gly Ser Tyr Glu Asn
 385 390 395 400

Trp Ile Asp Asn Gln Arg Lys Gln Phe Asp Lys Gln Lys Tyr Thr
 405 410 415

Lys Glu Ile Ser Asp Gly Gly Arg Lys Lys Arg Ala Val Gly Gly
 420 425 430

Thr Thr Lys Tyr Glu Gly Tyr Glu Lys Ser Phe Tyr Glu Lys Leu Lys
 435 440 445

Asn Asp Gly Tyr Gly Thr Val Asp Ala Phe Leu Gly Leu Leu Asn Asn
 450 455 460

Glu Lys Ala Cys Lys Asp Ile Thr Asp Gly Gly Lys Ile Asn Phe Lys
 465 470 475 480

Glu Val Asn Ser Gly Gly Val Val Gly Gly Ser Gly Gly Thr
 485 490 495

Ser Gly Ala Ser Gly Thr Asn Asp Glu Asn Lys Gly Thr Phe Tyr Arg
 500 505 510

Ser Glu Tyr Cys Gln Pro Cys Pro Asp Cys Gly Val Gln His Lys Gly
 515 520 525

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Gly Asn Gln Trp Glu Arg Lys Thr Lys Val Lys Lys Met Arg Trp Ser
 530 535 540

Lys Leu Tyr Lys Pro Ile Asn Gly Lys Met Val Leu Leu Leu Lys Ser
 545 550 555 560

Leu Lys Val Val Lys Asp Met Met Ile Leu Lys Lys Asn Trp Lys Glu
 565 570 575

Phe Cys Leu Thr Gln Asn Ser Ser Asp Gly Ser Val Gly Ser Val Val
 580 585 590

Thr Thr Gly Ala Ser Gly Gly Asn Ser Glu Lys Lys Glu Leu Tyr Asp
 595 600 605

Glu Trp Lys Cys Tyr Lys His Asn Glu Val Gln Lys Val Asn Val Gln
 610 615 620

Gly Glu Val Glu Glu Asp Asp Asp Glu Leu Lys Gly Ala Gly Gly Leu
 625 630 635 640

Cys Ile Leu Pro Asn Pro Lys Lys Asn Lys Glu Val Ser Glu Ala Lys
 645 650 655

Ser Gln Asn Asn His Ala Asp Ile Gln Lys Thr Phe His Asp Phe Phe
 660 665 670

Tyr Tyr Trp Val Ala His Met Leu Lys Asp Ser Ile His Trp Arg Thr
 675 680 685

Lys Arg Leu Lys Ser Cys Ile Ser Asp Gly Lys Thr Met Lys Cys Arg
 690 695 700

Asn Gly Cys Asn Lys Lys Cys Asp Cys Phe Glu Lys Trp Val Lys Gln
 705 710 715 720

Lys Glu Thr Glu Trp Lys Pro Ile Lys Asp His Phe Lys Thr Gln Glu
 725 730 735

Gly Ile Pro Glu Gly Tyr Tyr Phe Thr Thr Leu Glu Leu Ile Leu Lys
 740 745 750

Leu Gln Phe Leu Lys Glu Asp Thr Glu Glu Asn Thr Glu Asn Ser Leu
 755 760 765

Asp Ala Glu Glu Ala Glu Glu Leu Lys His Leu Gln Lys Ile Leu Lys
 770 775 780

Leu Glu Asn Glu Asn Asn Leu Ala Val Val Asn Ala Gly Thr Glu Gln
 785 790 795 800

Lys Thr Leu Met Asp Lys Leu Leu Asn His Glu Leu Asn Asp Ala Thr
 805 810 815

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Lys Cys Lys Asp Cys Pro Leu Pro Glu Glu Asp Lys Ser Arg Gly Arg
 820 825 830

Ser Ala Asp Pro Ser Pro Asp Ile Phe Ile Pro Arg Pro Glu Glu Lys
 835 840 845

-

Glu Asp Asp Glu Asn Glu Asp Asp Asp Glu Asp Val Arg Asp Asp
 850 855 860

Glu Glu Thr Ala Lys Glu Thr Thr Glu Gly Ser Ala Thr Asp Thr Thr
 865 870 875 880

Thr Ser Leu Asp Val Cys Pro Ile Val Gly Lys Val Leu Thr Lys Asp
 885 890 895

Asn Glu Ser Leu Gln Asp Ala Cys Ser Leu Lys Tyr Gly Gly Asn Asn
 900 905 910

Ser Arg Leu Gly Trp Arg Cys Val Thr Pro Ser Gly Glu Pro Thr Thr
 915 920 925

Ser Ser Asp Lys Asn Gly Ala Ile Cys Val Pro Pro Arg Arg Arg Arg
 930 935 940

Leu Tyr Ile Lys Lys Ile Val Asp Trp Ala Thr Lys Thr Glu Ser Pro
 945 950 955 960

Gln Ala Ser Gly Ser Glu Ala Ser Ser Thr Ser Gly Ser Thr Thr Pro
 965 970 975

Pro Asp Ser Lys Glu Ala Leu Leu Lys Ala Phe Val Glu Ser Ala Ala
 980 985 990

Ile Glu Thr Phe Phe Leu Trp His Arg Tyr Lys Glu Glu Lys Lys Ala
 995 1000 1005

Val Ala Gln Glu Gly Ala Gly His Gly Leu Pro Arg Val Glu Glu Gly
 1010 1015 1020

Ser Pro Glu Tyr Asp Pro Glu Asp Lys Leu Lys Glu Gly Lys Ile Pro
 1025 1030 1035 1040

Asp Gly Phe Leu Arg Gln Met Phe Tyr Thr Leu Gly Asp Tyr Arg Asp
 1045 1050 1055

Ile Leu Phe Ser Gly Ser Asn Asp Thr Thr Ser Val Ser Lys Asp Thr
 1060 1065 1070

Pro Ser Ser Ser Asn Asp Asn Leu Lys Asn Ile Val Leu Leu Ala Ser
 1075 1080 1085

Gly Ser Thr Glu Gln Glu Arg Glu Lys Met Asn Lys Tyr Lys Glu Ile
 1090 1095 1100

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Lys Asn Phe Arg Lys Cys Ser Thr Glu Arg Ser Ala Pro Asn Leu Val
 1105 1110 1115 1120

Ser His Pro Gln Thr Trp Trp Glu Asn Asn Gly Lys Tyr Ile Trp His
 1125 1130 1135

Gly Met Val Cys Ala Leu Thr Ser Lys Asp Lys Ile Ala Lys Gly Val
 1140 1145 1150

Glu Lys Lys Pro Gln Lys Ile Glu Asn Pro Glu Asn Leu Trp Asp Glu
 1155 1160 1165

Ala Asn Lys Lys Pro Lys Pro Pro Gln Tyr Gln Tyr Thr Asn Val Lys
 1170 1175 1180

Leu Asp Glu Asn Ser Gly Thr Ser Pro Arg Thr Thr Gln Thr Gln Ala
 1185 1190 1195 1200

Ser Ser Asp Asn Thr Pro Thr Thr Leu Thr His Phe Val Lys Arg Pro
 1205 1210 1215

Thr Tyr Phe Arg Trp Phe Glu Glu Trp Gly Glu Ser Phe Cys Arg Glu
 1220 1225 1230

Arg Lys Lys Arg Leu Lys Gln Ile Lys Val Asp Cys Lys Val Glu Asn
 1235 1240 1245

Gly Asp Val Gly Arg Cys Ser Gly Asp Gly Glu Ala Cys Asp Ser Ile
 1250 1255 1260

Ser Thr His Asp Tyr Ser Thr Val Pro Ser Phe Asn Cys Pro Gly Cys
 1265 1270 1275 1280

Gly Lys His Cys Ser Ser Tyr Arg Lys Trp Ile Glu Arg Lys Lys Ile
 1285 1290 1295

Glu Phe His Lys Gln Ser Asn Ala Tyr Gly Gln Gln Lys Thr Asp Ala
 1300 1305 1310

Thr Arg Asn Asn Gly Asn Thr Phe Asp Lys Glu Phe Cys Lys Thr Leu
 1315 1320 1325

Glu Thr Trp Pro Asp Ala Ala Lys Phe Leu Glu Arg Leu Lys Asn Gly
 1330 1335 1340

Pro Cys Lys Thr Asn Lys Glu Tyr Gly Asp Asp Ile Asp Phe Glu
 1345 1350 1355 1360

Lys Asp Ser Lys Thr Phe Gln His Thr Glu Tyr Cys Gly Pro Cys Pro
 1365 1370 1375

Lys Phe Lys Thr Asn Cys Gln Asn Gly Asn Cys Gly Val Ser Gly Leu
 1380 1385 1390

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Asn Gly Asn Cys Asp Gly Asp Lys Ser Ile Asp Ala Lys Glu Ile Ala
 1395 1400 1405
 Lys Met Arg Ser Ser Thr Thr Asp Val Val Met Arg Val Ser Asp Asn
 1410 1415 1420
 Asp Thr Asn Thr Phe Glu Gly Asp Asp Leu Lys Asp Ala Cys Gln His
 1425 1430 1435 1440
 Ala Asn Ile Phe Lys Gly Ile Arg Lys Asp Val Trp Lys Cys Gly Tyr
 1445 1450 1455
 Val Cys Gly Val Asp Ile Cys Glu Gln Thr Asn Ile Asn Glu Arg Thr
 1460 1465 1470
 Asp Gly Lys Glu Tyr Ile Gln Ile Arg Ala Leu Phe Lys Arg Trp Val
 1475 1480 1485
 Glu Asn Phe Leu Glu Asp Tyr Asn Lys Ile Asn Asp Lys Ile Ser His
 1490 1495 1500
 Cys Ile Lys Lys Gly Glu Gly Ser Lys Cys Ile Asn Gly Cys Glu Lys
 1505 1510 1515 1520
 Asn Ser Lys Cys Leu Glu Lys Trp Ile Glu Lys Lys Ile Ala Glu Trp
 1525 1530 1535
 Glu Asn Ile Lys Lys Arg Phe Asn Asp Gln Tyr Glu Asn Lys Asp Gln
 1540 1545 1550
 Pro Asp Tyr Asn Val Lys Ser Ile Leu Glu Leu Ile Pro Lys Ile
 1555 1560 1565
 Ala Val Val Asn Asp Gln Asp Asn Val Ile Lys Leu Cys Val Phe Glu
 1570 1575 1580
 Asn Ser Lys Gly Cys Thr Leu Ile Ser Asn Thr Gln Asn Asn Lys Glu
 1585 1590 1595 1600
 Asn Asp Ala Ile Asp Cys Met Lcu Lys Lys Leu Gly Val Lys Ala Lys
 1605 1610 1615
 Asn Cys Pro Gly Lys Pro Ser Gly Glu Lys Gln Ser Asp Cys Lys Glu
 1620 1625 1630
 Pro Pro Pro Leu Pro Asp Glu Glu Asp Gln Asn Pro Glu Glu Asn Thr
 1635 1640 1645
 Leu Glu Pro Pro Lys Phe Cys Pro Pro Thr Thr Gln Pro Pro Glu Glu
 1650 1655 1660
 Lys Gly Gly Glu Thr Cys Gly Asn Lys Glu Glu Lys Lys Asp Glu Lys
 1665 1670 1675 1680

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Lys Glu Glu Ser Glu Glu Pro Ala Lys Glu Glu Ser Gly Pro Ala Ala
 1685 1690 1695

Glu Glu Pro Ala Pro Thr Ala Glu Ser Glu Glu Thr Glu Thr Asn Phe
 1700 1705 1710

Pro Glu Pro Pro Gly Thr Gly Pro Ala Ala Pro Pro Ser Thr Pro Ala
 1715 1720 1725

Pro Pro Thr Pro Asp Thr Pro Pro Leu Arg Pro Gln Ala Asp Glu
 1730 1735 1740

Pro Phe Asp Ser Thr Ile Leu Gln Thr Thr Ile Pro Phe Gly Val Ala
 1745 1750 1755 1760

Leu Ala Leu Gly Ser Ile Ala Phe Leu Phe Leu Lys Lys Lys Thr Lys
 1765 1770 1775

Ala Ser Val Gly Asn Leu Phe Gln Ile Leu Gln Ile Pro Lys Ser Asp
 1780 1785 1790

Tyr Asp Ile Pro Thr Leu Lys Ser Ser Asn Arg Tyr Ile Pro Tyr Val
 1795 1800 1805

Ser Asp Arg Tyr Lys Gly Lys Thr Tyr Ile Tyr Met Glu Gly Asp Ser
 1810 1815 1820

Asp Glu Asp Lys Tyr Ala Phe Met Ser Asp Thr Thr Asp Val Thr Ser
 1825 1830 1835 1840

Ser Glu Ser Glu Tyr Glu Glu Leu Asp Ile Asn Asp Ile Tyr Val Pro
 1845 1850 1855

Gly Ser Pro Lys Tyr Lys Thr Leu Ile Glu Val Val Leu Glu Pro Ser
 1860 1865 1870

Gly Asn Asn Thr Thr Ala Ser Gly Lys Asn Thr Pro Ser Asp Thr Arg
 1875 1880 1885

Asn Asp Ile Gln Asn Asp Gly Ile Pro Ser Ser Lys Ile Thr Asp Asn
 1890 1895 1900

Glu Trp Asn Gln Leu Lys Lys Glu Phe Ile Ser Asn Met Leu Gln Asn
 1905 1910 1915 1920

Gln Pro Asn Asp Val Pro Asn Asp Tyr Thr Ser Gly Asn Ser Ser Thr
 1925 1930 1935

Asn Thr Asn Ile Thr Thr Ser Arg His Asn Val Asp Asn Asn Thr
 1940 1945 1950

Asn Thr Thr Met Ser Arg Asp Asn Met Glu Glu Asn Leu Leu Leu Pro
 1955 1960 1965

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Ser Ile His Asp Gly Asn Leu Tyr Ser Gly Glu Glu Tyr Ser Tyr Asn
 1970 1975 1980

 Val Asn Met Val Asn Ser Met Asn Asp Ile Pro Ile Asn Arg Asp Asn
 1985 1990 1995 2000

 Asn Val Tyr Ser Gly Ile Asp Leu Ile Asn Asp Ser Leu Ser Gly Gly
 2005 2010 2015

 Lys Pro Ile Asp Ile Tyr Asp Glu Val Leu Lys Arg Lys Glu Asn Glu
 2020 2025 2030

 Leu Phe Gly Thr Glu Asn Thr Lys Arg Thr Ser Thr Gln Asn Val Ala
 2035 2040 2045

 Lys Thr Thr Asn Ser Asp Pro Ile His Asn Gln Leu Glu Leu Phe His
 2050 2055 2060

 Lys Trp Leu Asp Arg His Arg Asp Met Cys Glu Lys Trp Lys Asn Lys
 2065 2070 2075 2080

 Glu Asp Ile Leu Asn Lys Leu Lys Glu Glu Trp Asn Lys Glu Asn Ile
 2085 2090 2095

 Asn Asn Ser Gly Lys Thr Tyr Asn Ser Asp Asn Lys Pro Ser His Asn
 2100 2105 2110

 His Val Leu Asn Thr Asp Val Ser Ile Gln Ile Asp Met Asp Asn Pro
 2115 2120 2125

 Lys Thr Lys Asn Glu Ile Thr Asn Met Asp Thr Asn Gln Asp Lys Ser
 2130 2135 2140

 Thr Met Asp Thr Ile Leu Asp Asp Leu Glu Lys Tyr Asn Asp Pro Tyr
 2145 2150 2155 2160

 Tyr Tyr Asp Phe Tyr Glu Asp Asp Ile Ile Tyr His Asp Val Asp Val
 2165 2170 2175

 Glu Lys Ser Ser Met Asp Asp Ile Tyr Val Asp His Asn Asn Val Thr
 2180 2185 2190

 Ser Asn Asn Met Asp Val Pro Thr Lys Met His Ile Glu Met Asn Ile
 2195 2200 2205

 Val Asn Asn Lys Lys Glu Ile Phe Glu Glu Glu Tyr Pro Ile Ser Asp
 2210 2215 2220

 Ile Trp Asn Ile
 2225